

CONFORMATIONAL ASPECTS OF VALINOMYCIN
BY ^1H AND ^{13}C NUCLEAR MAGNETIC RESONANCE

Thesis by
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To my friends

I've spent some time in study,
Oh, I've taken my degrees;
I've memorized my formulae,
My A's and B's and C's,
But what I know came long ago
And not from such as these,
And I'm going to be a country girl again.

-Buffy Ste. Marie

ABSTRACT

The conformation of valinomycin and its complex with potassium ion has been investigated by spectroscopic means and a plausible structure for the complex has been put forward. The essential features of the complex structure are consistent with the spectral data and the conformation in the crystal state. Inconsistencies between the spectral observations and crystal structure analysis of non-complexing valinomycin may be resolved if a rapid conformation equilibrium in solution is postulated; evidence for this process is discussed. The characteristics of valinomycin are compatible with an independent mechanism of ion transport.

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Introduction

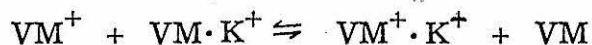
Among the most interesting of the properties of natural membranes are their selective permeability to various solutes, and, related to their selective cation permeability, their unusual electrical characteristics. These effects are manifested in the resting state of biological membrane systems, especially nervous systems, in which a concentration differential is maintained of approximately 15 : 1 outside vs. inside with respect to Na^+ and approximately 1 : 50 outside vs. inside with respect to K^+ (Katz, 1966).

Propagation of nerve impulses involves disruption of this resting (equilibrium) potential by the flow of ionic species, notably Na^+ and K^+ , across the lipid barrier of the nerve axon membrane in a direction perpendicular to the direction of impulse propagation, but it remains obscure exactly how this ready flow of highly polar species through a distinctly hydrophobic medium is accomplished; the same question arises in contemplation of mechanisms for the "sodium pump" which maintains the appropriate membrane resting polarization. There have been three basic hypotheses as to this means of transport, implicating, as structures responsible for ion flow through the membrane, isolated carrier species (Haynes, 1969; Ohnishi and Urry, 1969) organized pores of flux-mediating molecules or membrane structures modified locally by interaction with particular molecules to form areas of ready penetration (Duax, 1972). The discovery that certain antibiotic molecules were capable of mediating ion transport

across natural and artificial membranes (Pressman, 1968 and references therein; Mueller and Rudin, 1967) provided model systems with properties in many ways similar to those exhibited by the nerve cell membrane system.

Many of these ionophores have differential affinities for various cations and of them the most selective is valinomycin (VM) which has an affinity for K^+ nearly 300 times as great as for Na^+ (Mueller and Rudin, 1967a). Produced by Streptomyces fulvissimus, valinomycin is a cyclic trimer of the four-residue subunit (l-lactate-l-valine-d- α -hydroxy-isovalerate-d-valine). The noteworthy features of this sequence are the alternating ester and amide linkages in the backbone, the exclusively hydrophobic nature of the side chains, and the potential for three-fold symmetry offered by the trimeric primary structure. The unique transport selectivity of the molecule and the fact that no three-dimensional structure was known (although conjectures had been made; Warner, 1966; Mueller and Rudin, 1967a) made it an interesting species for investigation by spectroscopic means, with the object of ascertaining the molecular conformation and from that seeking to learn about the ion-selective and transport properties of the molecule. Nuclear magnetic resonance (nmr) spectroscopy was chosen as the chief means of investigation because of the great sensitivity of the method to changes in conformational parameters, its possibility of revealing dynamic aspects of the molecule's behavior and, not least, because of the convenient availability of sophisticated equipment and expertise.

In the interval since the start of this research, substantial progress toward understanding the behavior of valinomycin has been made by a number of groups and a brief review of past events is in order. The assignment of the ^1H nmr spectrum described in the following pages was soon found to be in agreement with that reported by Haynes and co-workers (1969) in experiments performed at lower field (14.1 Kguass). On the basis of measurement (1969) of the lactyl methyl line widths Haynes et al. concluded that the exchange process



was immeasurably slow in non-polar solvents, and from the lack of concentration effects on the spectrum, concluded that valinomycin did not aggregate substantially in solution. These conclusions were offered as arguments against a pore-forming mechanism for valinomycin-mediated K^+ transport, and in favor of an individual carrier complex; no tertiary structural information was reported.

Shortly thereafter, Ivanov et al. (1969) reported infrared (ir), optical rotatory dispersion (ORD) and circular dichroism (CD) data and proposed tertiary structures both for valinomycin alone and for the $\text{VM} \cdot \text{K}^+$ complex which provided substantial refinement on the structure described here. The nmr data presented by Ivanov et al. were in agreement with those obtained previously (Haynes et al., 1969) and described here, and the assignments were extended to distinguish between the d- and l-valyl amide and backbone methynyl resonances although no rationalization for this distinction was

offered. Ivanov et al. interpreted their ORD data in media of low polarity as indicating an equilibrium between a number of conformers, and from the observation of both H-bonded and non-H-bonded amide N-H and $>C=O$ stretching modes in the ir spectrum, concluded that a major contributor to the equilibrium was a conformer in which each amide carbonyl was involved in H-bonding with the proton of the neighboring amide group. A minor contributor to the equilibrium structure in non-polar media, increasing in importance as solvent polarity increased, was considered to be a structure in which some number of amide carbonyls were involved in hydrogen bonds to solvent molecules. In the complex with K^+ Ivanov et al. (1969) also concluded from the ir spectrum that the ester carbonyls were involved in an ion-dipole interaction and further that all amide protons were involved in H-bonds; from ORD spectra they concluded that no conformational equilibrium was present.

The spectroscopic conformational predictions were supported by an analysis of the crystal structure of the valinomycin- K^+ complex (Pinkerton et al., 1969) which showed that the depsipeptide backbone of the molecule adopted the three-fold symmetric "bracelet" configuration proposed for it (Ivanov, 1969), but which did not report side-chain orientations or atomic coordinates. Subsequent publications by other groups (Haynes et al., 1971; Ohnishi and Urry, 1969, 1970; Mayers and Urry, 1972) confirmed the spectroscopic and crystallographic conclusions and indicated the popularity of the problem; in fact highly similar structures for the valinomycin K^+ complex had been

determined independently by at least three groups (Ivanov, 1969; Ohnishi, 1969; Macmurchie, unpublished). Further information was sought from the spectrum of the ^{13}C resonances, and the assignments and spectral changes upon K^+ complex formation described here are in agreement with the recent work of Ohnishi et al. (1972) and Bystrov et al. (1972).

Observing spectroscopic indications of symmetry, most investigators (Ivanov, 1969; Pinkerton, 1969; Ohnishi and Urry, 1969; Mayers and Urry, 1971; Macmurchie, unpublished) had assumed for valinomycin alone, a conformation generally similar to that which it had been shown to adopt in the presence of K^+ , and so the crystallographic results of Duax et al. (1972), indicating an entirely asymmetric structure for the unoccupied antibiotic, are extremely interesting. If the conformation in solution is the same as in the crystal one must ask why certain of its features are not revealed in the nmr spectra; if the structures are not the same, he must decide which is most appropriate to the environment in which the molecule functions. It would be desirable to reconcile the results of both techniques, and the possibility of doing this as well as its implications for the behavior of the molecule will be discussed.

¹H spectrum: assignment

The ¹H nmr spectra of valinomycin (Fig. 1) and its complex with K⁺ were recorded using Varian HR-220, XL-100/15, HA-100, A-60 and T-60 spectrometers, with the 220 MHz instrument, by virtue of its greater resolution and sensitivity, being most frequently employed. Valinomycin was obtained from Cal Biochem, lot 860009 and used without further purification; all solvents were spectro-quality and tetramethylsilane (TMS) was used as an internal reference.

The ¹H resonances were assigned as indicated in Table 1 on the assumption that the spectrum could be interpreted in terms of a single four-residue subunit, i. e., each line was produced by the resonances of three magnetically equivalent nuclei; and according to the following rationale.

After equilibration of the sample in a solution of 10% D₂O/acetone the two doublets at lowest field (δ = 7.84, 7.89 ppm) were no longer present in the spectrum, and this fact, in addition to their appropriate chemical shifts, makes them attributable to the amide protons of the d- and l-valyl residues, these being the only protons considered likely to undergo exchange with the solvent. On elimination of the amide resonances, the two quartets at 3.99 ppm and 4.14 ppm collapsed to doublets, and these resonances therefore are assigned to the α -protons of the valyl residues. Inspection of the proton-proton couplings (Table 1) leads to the conclusion that the lower field amide resonance is coupled to the lower field α -proton resonance, but a choice as to which was the d- or l-isomer could not be made.

Figure 1: Valinomycin ^1H nmr spectrum at 220 MHz in CDCl_3 ; each trace is 500 Hz, continuous from high field at top.

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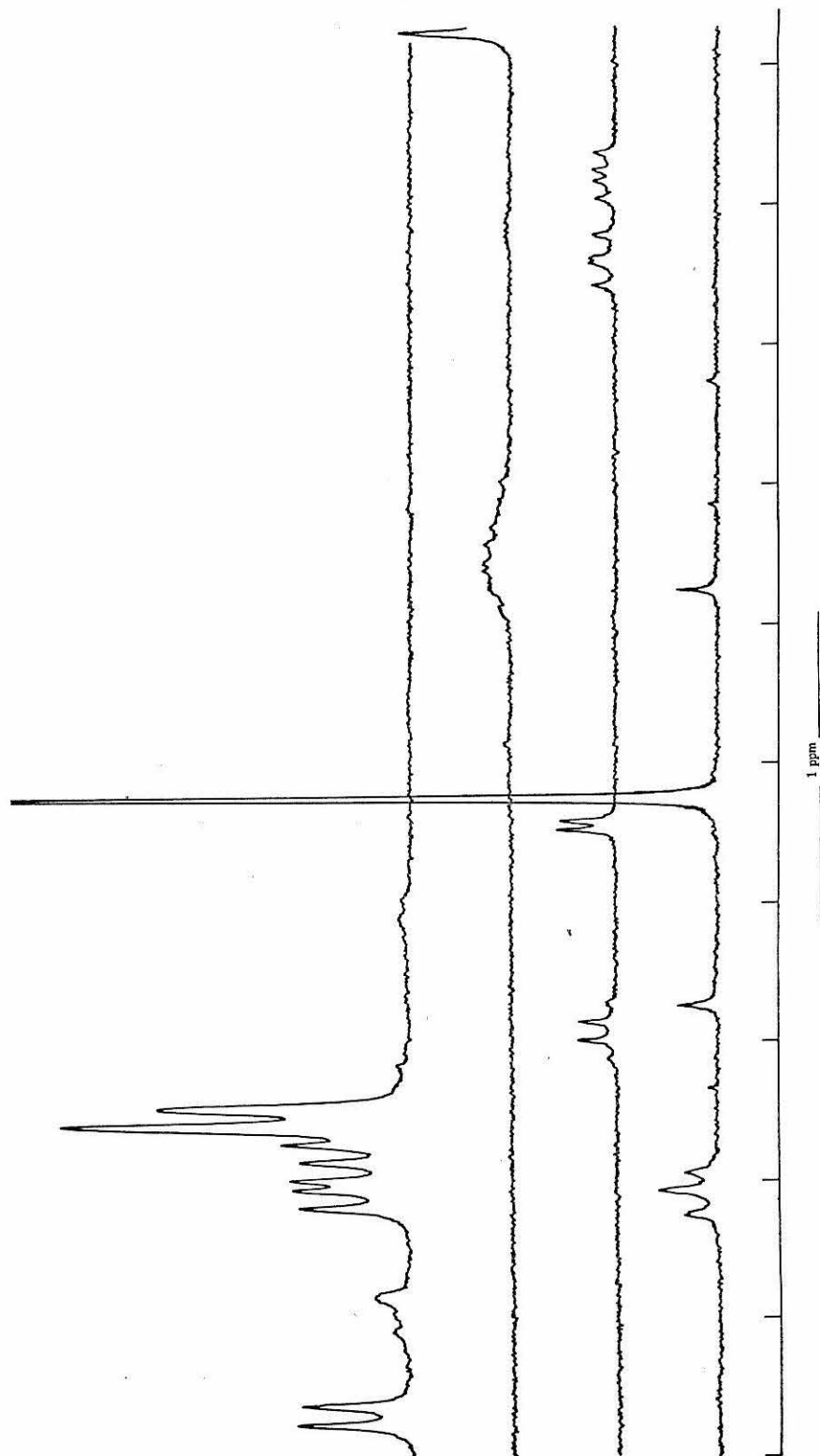


Table 1

¹H Resonance Assignments for Valinomycin in CDCl₃

δ ppm from TMS	description	coupling	integration	assignment *
0.96 - 1.11	six doublets	~ 7 Hz	223	d-Val l-val αHiv methyls
1.46	doublet	6.8 Hz	38	lactate methyls
~ 2.3	three multiplets		37	d-val l-val αHiv β-protons
3.99	two doublets	6.2, 10.1 Hz	11	l-val α-protons
4.14	two doublets	8.1, 10.0 Hz	11	d-val α-protons
5.02	doublet	2.8 Hz	11	αHiv α-protons
5.34	quartet	6.8 Hz	9	lactate α-protons
7.84	doublet	6.2 Hz	9	l-val amide protons
7.89	doublet	8.1 Hz	9	d-val amide protons

* distinction between d- and l-isomers is that of Ivanov et al. (1969).

The other α -proton resonances, at $\delta = 5.34$ ppm and $\delta = 5.02$ ppm, are therefore those of the lactyl and hydroxy iso-valeryl residues respectively, as indicated by their multiplicity. The β -proton resonances of the d- and l-valyl and hydroxyiso-valeryl residues are expected to be patterns of at least eight lines each, and these three groups compose the poorly resolved envelope at approximately 2.4 ppm from TMS. The lactyl methyl resonance at $\delta = 1.46$ ppm was assigned on the basis of its position separate from other resonances in the methyl region and its intensity, the single α Hiv α -proton giving rise to the only other doublet. The remaining methyl resonances, arising from the d- and l-valyl and hydroxyiso-valeryl residues, overlap one another extensively, as do the β -protons of the same residues, obscuring their assignments.

^1H spectrum: interpretation

The ^1H resonance spectrum affords a considerable amount of information and in a molecule such as valinomycin which lacks functional side chains, the most detailed of this information is frequently obtained from the study of proton-proton couplings; in valinomycin these couplings are all between protons bonded to adjacent carbons or nitrogens, i.e., three-bond couplings. For these it is possible to relate the magnitude of the coupling constant $^3J_{\text{HCCH}}$ to the dihedral angle between the bonds involving the two protons (Karplus, 1959). The Karplus equation for protons on adjacent carbons, as modified by Abraham and McLauchlan (1963) is:

$$^3J_{\alpha\beta} = 10.5 \cos^2 \theta - 0.28 \text{ Hz for } 0^\circ < \theta \leq 90^\circ \quad 1a$$

and

$$^3J_{\alpha\beta} = 13.7 \cos^2 \theta - 0.28 \text{ Hz for } 90^\circ < \theta \leq 180^\circ \quad 1b$$

In addition, Bystrov and co-workers (1969) have determined a similar relation for the $^3J_{\text{HNCH}}$ proton-proton couplings for amino acids:

$$^3J_{\text{HNCH}} = 8.9 \cos^2 \theta - 0.9 \cos \theta + 0.9 \sin^2 \theta \quad 2$$

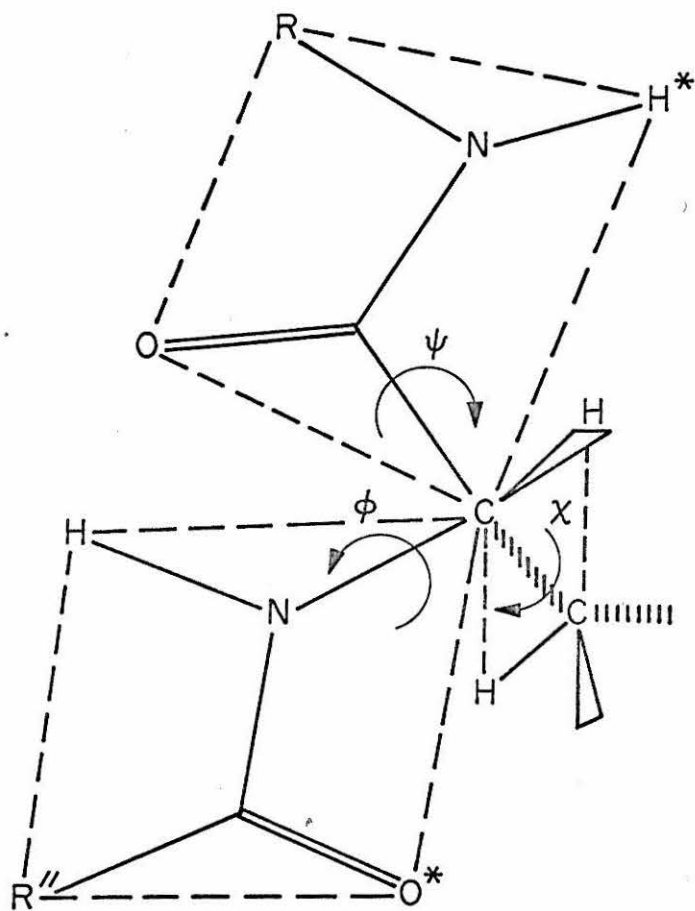
or

$$^3J_{\text{HNCH}} = 8.0 \cos^2 \theta - 0.9 \cos \theta + 0.9$$

Using these relations one may estimate dihedral angles ϕ and χ (Fig. 2) at various locations in both valinomycin and the complex with K^+ as indicated in Table 2. It is to be noted, however, that in general, four distinct angles of rotation can give rise to the same coupling constant, and that some other criteria are necessary if one is to choose among them. It has been shown by Gibbons et al. (1970) that a combination of spin-coupling data and conformational energy considerations (Scott and Scheraga, 1966) can reduce the number of possible configurations corresponding to a particular coupling constant; even so, an exact knowledge of the C_α -N dihedral angle (ϕ) is not sufficient to determine the backbone configuration of a peptide (Gibbons et al., 1970) in which the amide moiety is generally assumed to be planar, i.e., $\omega = 180^\circ$ (Corey and Pauling,

Figure 2: IUPAC-IUB peptide torsional angles;
 $\phi\psi = 0$ when H^* , O^* coincide.

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$$\phi = 180^\circ$$

$$\psi = 180^\circ$$

$$\chi = 180^\circ$$

Table 2

Dihedral Angles: VM and VM•K⁺ in CDCl₃

Residue		J		dihedral angle [*]	
		VM	VM•K ⁺	VM	VM•K ⁺
l-val	φ	6.2	4.6	180°	-144°
	χ	10.1	9.9	+153°	+151°
d-val	φ	8.1	5.1	- 39°	- 46°
	χ	10.0	9.6	+152°	+148°
lactate	χ	6.8	6.8	free rotation	
αHiv	χ	2.8	3.6	+123°	+127°

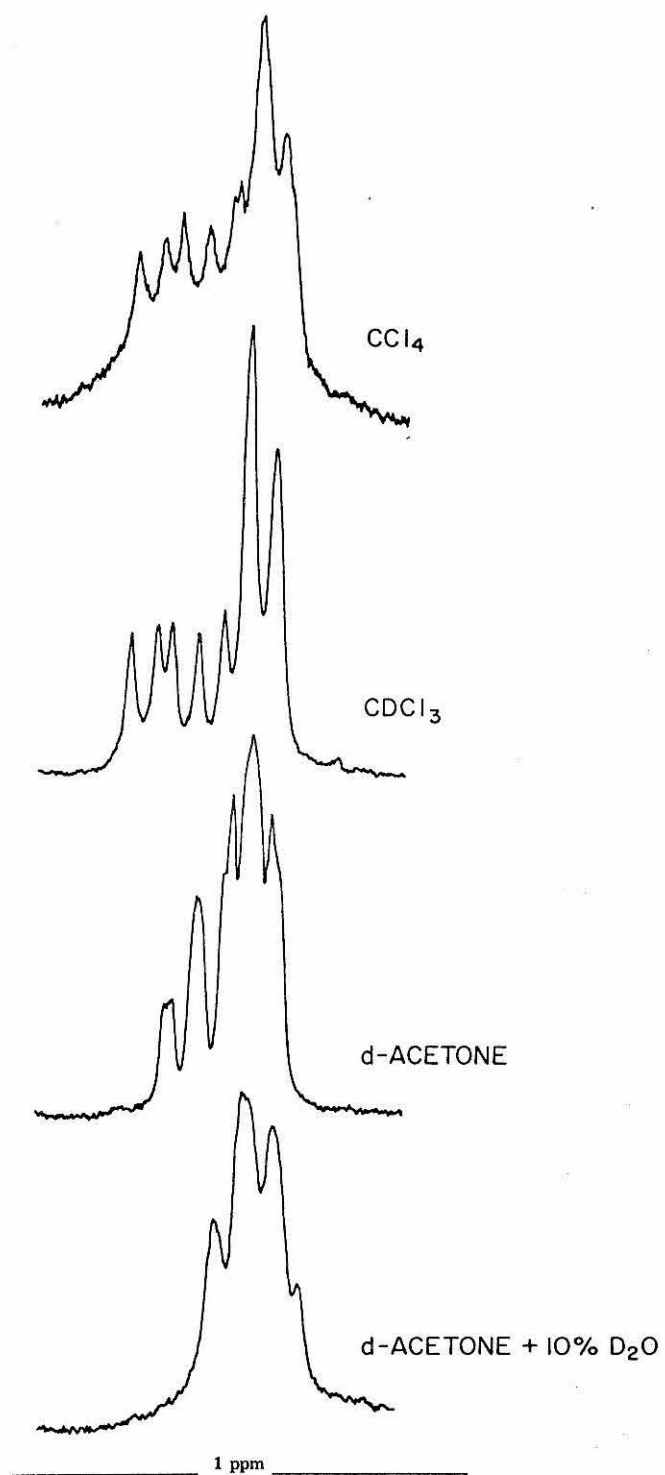
* chosen to minimize steric interference.

1953; Bystrov et al., 1970). Valinomycin, as a depsipeptide, enjoys the greater conformational freedom endowed by its ester linkages, and thus, in order to specify its conformation exactly, even more information is required.

The proton chemical shifts of valinomycin undergo changes on variation of solvent and on formation of the cation complex. This is particularly evident in the shifts of the methyl resonances, which in the absence of the cation, reflect the polarity of the medium as they progress from a poorly defined envelope in D_2O /acetone toward a relatively well-resolved system of doublets in CCl_4 (Fig. 3). As the factors giving rise to proton chemical shifts are of comparable importance (Carrington and McLachlan, 1967; Pople et al., 1959) it is tenuous to attribute these changes to a particular source, but it is reasonable to say that in chemically similar nuclei, similarity of chemical shift indicates similarity of chemical and magnetic environment, and that in this respect, the changes in methyl proton resonance positions with solvent polarity are indicative of a particularization of methyl-group environments as polarity decreases. This is to say that in the relatively polar D_2O /acetone (10% v/v) the methyl groups of the valyl and α -hydroxy-iso-valeryl side chains find themselves in relatively equivalent positions with respect to moieties that can affect their chemical shifts, whether they are solvent molecules or other groups on the antibiotic, but in the less polar solvents CCl_4 or $CHCl_3$, each methyl group experiences a more unique and possibly more restricted situation;

Figure 3: Valinomycin high-field methyl resonances,
showing effect of solvent polarity.

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this implies a higher degree of intramolecular organization in the less polar media, which is in agreement with the ORD studies of Ivanov et al. (1969).

In the case of the lower-field resonances, more specific effects on change of solvent were observed. Especially, it was observed that when the CDCl_3 solvent was saturated with D_2O , the amide proton resonance positions were altered to unequal degrees, with the result that these resonances, which had originally appeared as a pair of doublets, now occurred closer together, appearing almost as a triplet. This result was interpreted as indicating different hydration properties of the amides themselves, a conclusion that has been confirmed by Haynes et al. (1969) and by studies involving the temperature dependence of these resonances (Ohnishi and Urry, 1969); this conclusion is also in accord with that of Ivanov et al. (1969) that, depending upon solvent, one set of valine amide protons may form intramolecular H-bonds, while the other amide protons form H-bonds to solvent.

Proton resonance positions were also observed to experience alterations on formation of the cation complex. The chemical shifts of the methyl resonances are nearer to those of valinomycin in non-polar than in polar solvent, and are less solvent-dependent than those of the free antibiotic; this again is taken as an indication of structural integrity and of uniqueness of environment of those protons. The lower field resonances undergo changes in chemical shift as indicated in Table 3. It was observed that the resonances of the

Table 3

¹H Resonance Shifts on Complex Formation
in CDCl₃

<u>Resonance</u>	<u>Δppm</u>
lac Me	-0.10
l-Val(α)	+0.27
d-val(α)	+0.31
αHiv(α)	-0.43
lac(α)	-0.39
l-val(NH)	-0.50
d-val(NH)	-0.57

hydroxy acids occurred to lower field in the complex than in the free species, while those of the amino acids were shifted to higher field. Paramagnetic shifts in the presence of the electric field of the cation might be predicted (Emsley et al., 1965; Carrington and McLachlan, 1967), but the upfield shifts of the valine α -protons are more interesting, in that these protons are alpha to the carbonyls which are involved in the ion-dipole interaction (p. 21), an argument based simply on electronic induction would predict shifts to lower field; thus it is apparent that additional factors, presumably arising from conformational changes, are contributing to chemical shift changes on complex formation. These effects may be related to changes seen in the ^{13}C nmr spectrum, to be discussed subsequently.

Ir spectrum of Valinomycin:

The infrared absorption spectrum of valinomycin (Fig. 4a) is well-resolved and the depsi-peptide nature of the molecule is evidenced by the presence of both ester and amide carbonyl stretching modes, noted in Table 4; other salient features of the ir spectrum include the N-H stretching band at 3320 cm^{-1} and the alkyl oxygen stretching absorptions at 1185 cm^{-1} and 1132 cm^{-1} . On formation of the $\text{VM}\cdot\text{K}^+$ complex the spectrum was altered noticeably (Fig. 4c) with the most striking changes occurring in the ester bands. The ester $>\text{C}=\text{O}$ stretching mode was sharpened and decreased in frequency from 1755 cm^{-1} to 1738 cm^{-1} , while the alkyl oxygen stretching bands were present at decreased intensity; the amide carbonyl frequencies on the other hand were largely unaffected by complex formation, with only a slight sharpening of the peaks taking place. These observations are consistent with polarization of the ester carbonyls by a nearby positive charge (Nakanishi, 1962) and strongly implicated the valine residues in the interaction with the metal cation. This conclusion was also reached by Ivanov et al. (1969), who in addition observed the disappearance of shoulders on the N-H stretching band (ca. 3390 cm^{-1}) and the amide C=O band (1678 cm^{-1}) on complex formation and attributed these effects to constraint of all the amide protons in intramolecular H-bonds.

Implications for Valinomycin structure:

As was indicated previously, the nmr spectra, while pro-

Figure 4: Infrared absorption spectra of
a) valinomycin
b) valinomycin-KSCN, 10:4
c) valinomycin-KSCN, 1:1

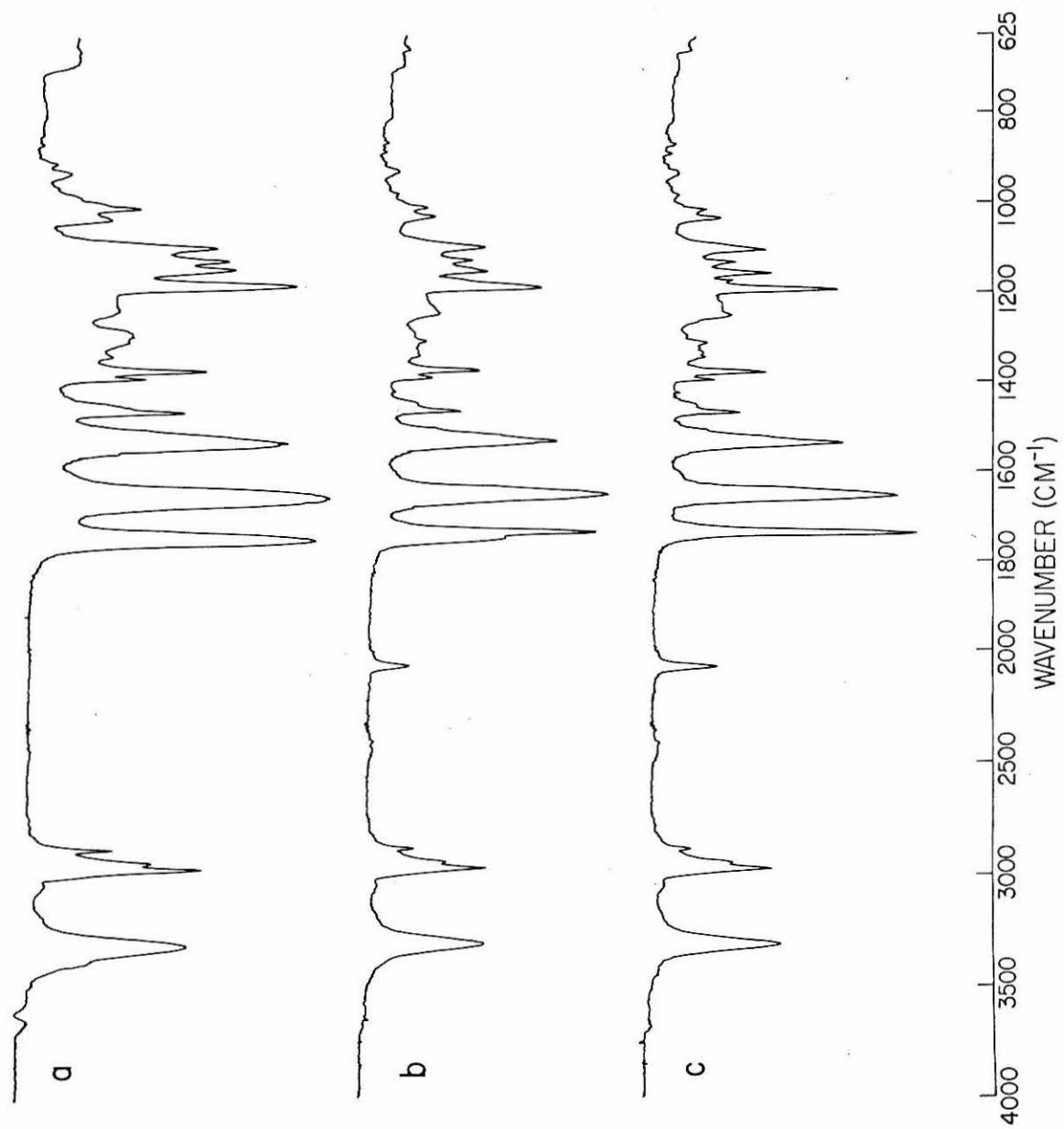


Table 4

Infrared Spectral Assignments

$\bar{\nu}$ (cm ⁻¹) intensity		assignment
native valinomycin		
3310	m	$>\text{N}-\text{H}$ stretch
1755	s	$>\text{C}=\text{O}$ (ester) stretch
1660	s	$>\text{C}=\text{O}$ (amide) stretch
1540	ms	$>\text{N}-\text{H}$ deformation
1185	ms	$\begin{array}{c} \diagup \\ \text{O} \\ \diagdown \end{array}$ (ester) asym. stretch
1151	m	gem dimethyl skeletal
1132	m	$\begin{array}{c} \diagdown \\ \text{O} \\ \diagup \end{array}$ (ester) sym. stretch
1103	m	gem dimethyl skeletal

viding useful information about side-chain orientation and the backbone angle ϕ were not by themselves sufficient to define a structure for valinomycin. Knowledge that the ester carbonyls were apparently uniquely involved in cation stabilization, however, substantially reduced the number of conformational choices, and, bearing in mind the apparent symmetry of the molecule as indicated by the nmr spectra, the six participating carbonyls were most logically located in an octahedral arrangement, which constrained the backbone of the molecule to a serpentine configuration enclosing the ion. Using space-filling models, it was possible to assemble a structural representation of the $VM \cdot K^+$ complex which was consistent with the spectral data and allowed coordination of the metal ion by the six ester moieties (Fig. 5). This structure resembles in some of its features that of the nonactin- K^+ complex (Kilbourn et al., 1967) which was described as resembling the seam on a tennis ball; in this context, the valinomycin complex resembles one and one-half tennis ball seams, with a coordinating carbonyl group at each bend. This arrangement provides a hydrophilic enclosure for the metal ion and a hydrophobic exterior suitable for situation in a lipid environment, as was also found to be the case for nonactin. Incorporation of the intramolecular H-bonds proposed by Ivanov et al. (1969) further constrains the structure and these constraints allowed Ivanov and co-workers, on the basis of an independent study of $^3J_{HNCH}$ coupling constants (Bystrov et al., 1969), to decide between two possible structures each with the same general features as the one

Figure 5: Proposed non-hydrogen-bonded
VM·K⁺ structure (Macmurchie, unpublished).

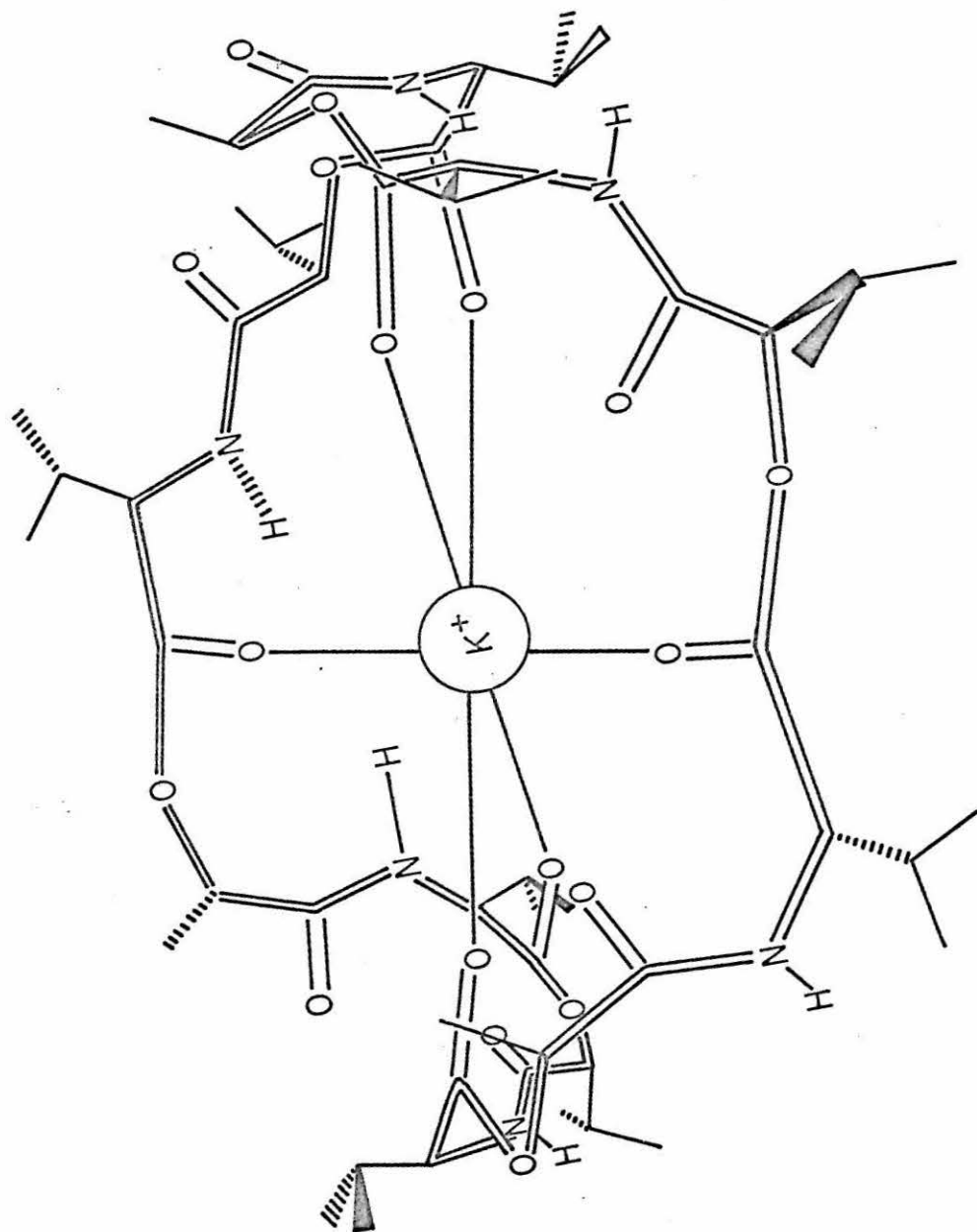
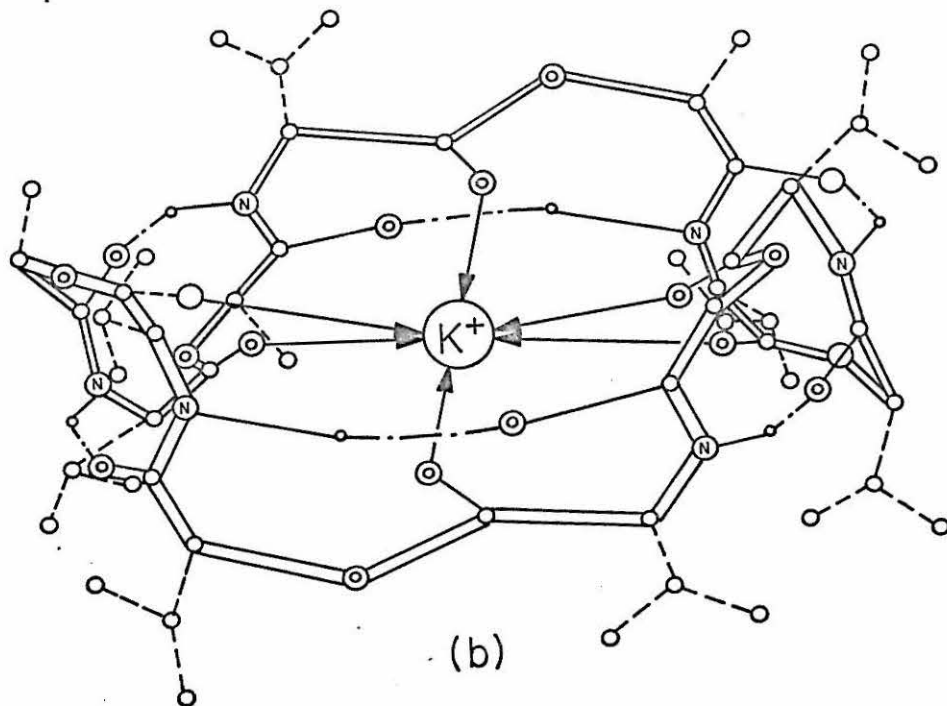
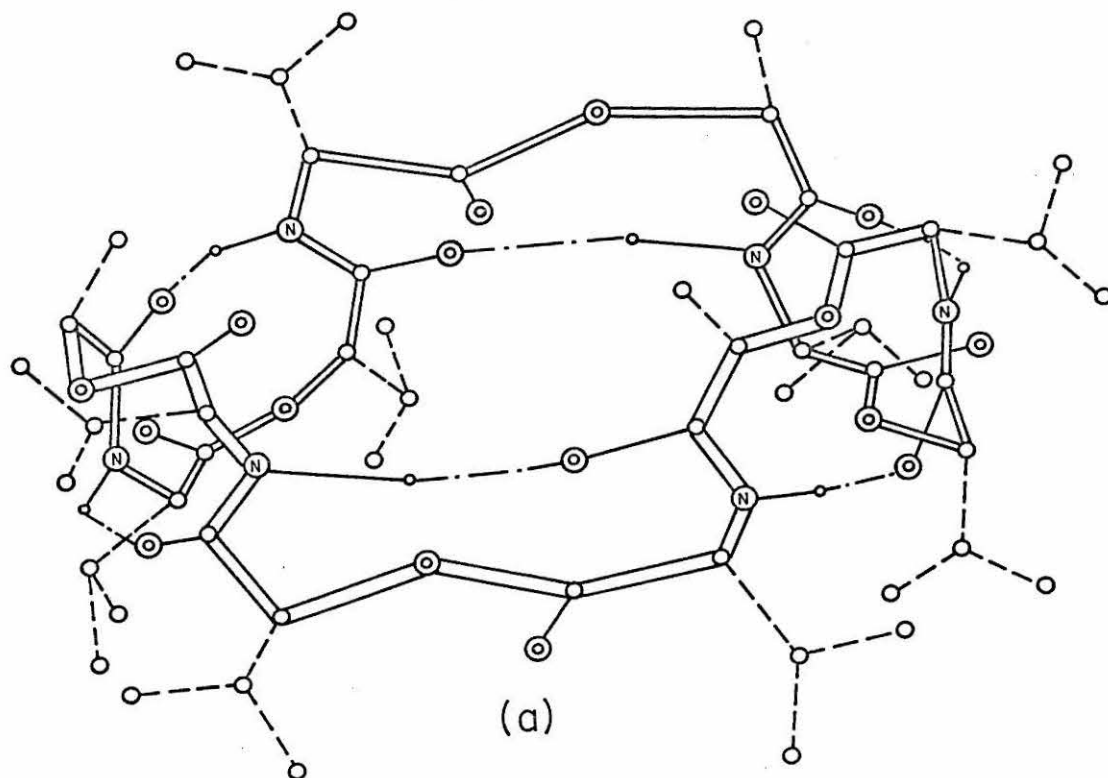


Figure 6: Proposed structures of:

a) valinomycin

b) valinomycin \cdot K⁺

(Ivanov et al., 1969).



in Fig. 5; their choice (Fig. 6) which was later confirmed by the X-ray analysis of Pinkerton et al. (1969), is the inside-out, H-bonded counterpart to that shown in Fig. 5.

^{13}C nmr spectrum:

The use of ^{13}C nmr offers several advantages in comparison to ^1H nmr: a smaller number of resonances is usually observed, the chemical shifts are larger (and thus more sensitive to subtle effects), and information is obtained directly from the framework of the molecule, tending to minimize intermolecular effects on the spectrum. The method has the disadvantages of the low sensitivity of the ^{13}C nucleus (1.6% of that of protons) and the fact that the natural abundance of ^{13}C is only 1.1%, but technical advances, especially the use of Fourier transform nmr, and the benefit of the Overhauser effect on decoupling from protons, can aid in overcoming these difficulties. In addition, recent progress in the correlation of ^{13}C chemical shifts with substituent effects has allowed the prediction of various resonance positions (Grant and Paul, 1964; Horsley et al., 1969; Gibbons et al., 1970). In the following pages the ^{13}C resonance positions are compared with those calculated and with those of the ^1H resonances in order to derive additional information about the VM - VM $\cdot\text{K}^+$ system.

Valinomycin was obtained from Cal Biochem (Lot 860021 and used without further purification; solvents were Matheson, Coleman and Bell spectroquality. Spectra were recorded using a Varian XL-100/15 spectrometer in the Fourier Transform mode

with noise decoupling from protons and employing a ^2H field-frequency lock signal from solvent or, in the case of non-deuterated solvent, from external $^2\text{H}_2\text{O}$. Tetramethylsilane was used as an internal reference. Preliminary experiments were performed using a 14.1 K gauss instrument made available through the kindness of the Department of Chemistry, University of California, Berkeley.

As was the case with the ^1H spectrum, the number of resonances observed in the ^{13}C spectrum (Fig. 7) corresponded to the number of carbon atoms in one of valinomycin's three subunits, reinforcing the inference that each chemically identical part of the molecule senses an identical magnetic environment, and the assignments (Table 5) were again made on that basis. The group of four resonances at lowest field was readily identified as those of the carbonyls by virtue of their characteristic chemical shifts and low intensities as a result of lack of nuclear Overhauser enhancement (Kuhlman and Grant, 1968). Assignment of the individual peaks was more difficult. Following preliminary experiments in acetone- d_6 , it was observed that the two carbonyl resonances at highest field were somewhat broader and lower in peak height than the two at lowest field, an effect that could be attributed to relaxation by a nearby quadrupolar nucleus (Pople et al., 1959; Emsley et al., 1965). Thus, the

Figure 7: ^{13}C FT nmr spectrum of valinomycin in
at 25.2 KHz. ^1H noise decoupled, spectrum
width 5 KHz.

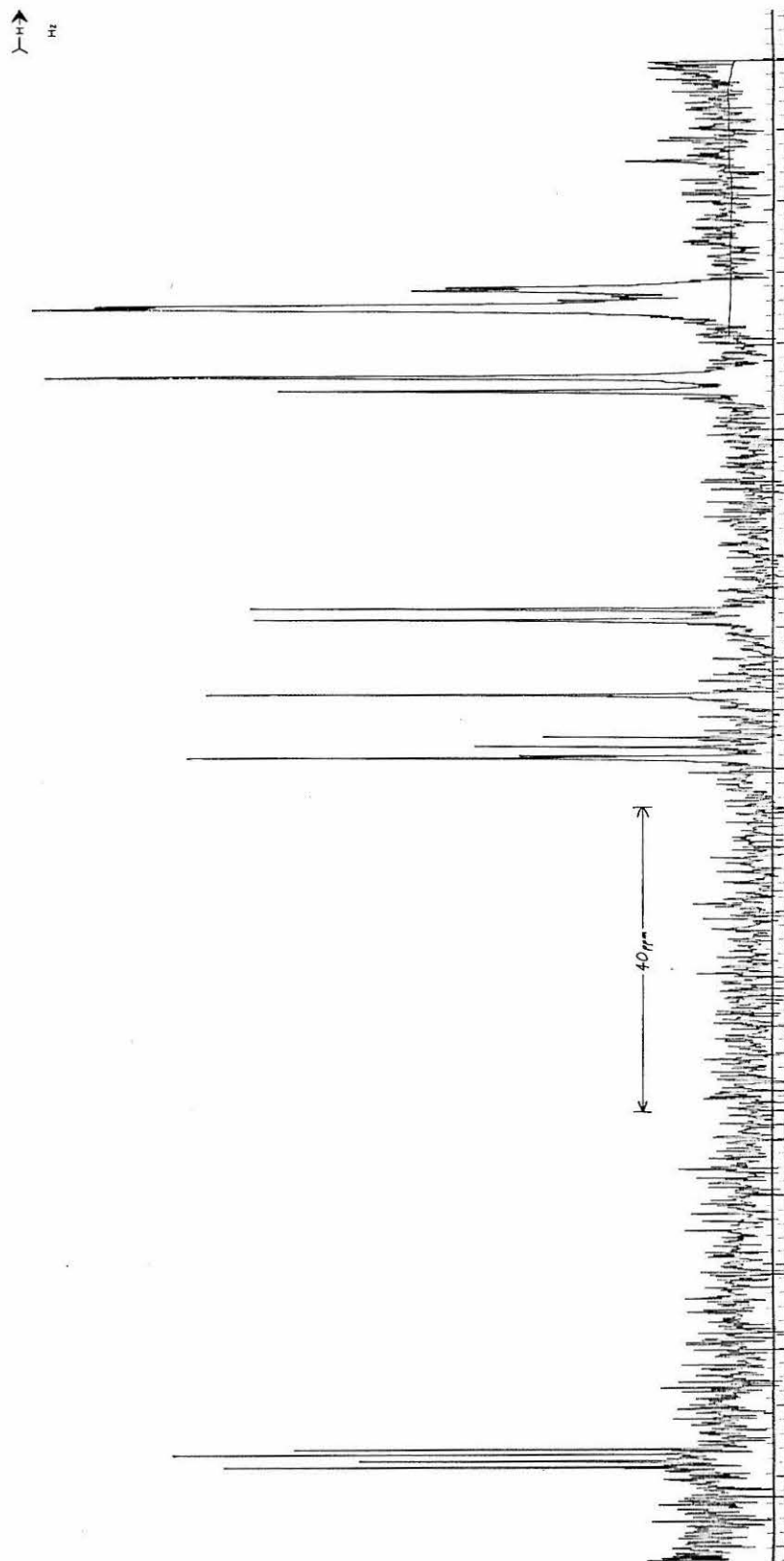


Table 5

Solvent Dependence of Valinomycin ^{13}C Chemical Shifts
(ppm from TMS)

resonance*	Solvent				calc.
	MeOH	Me ₂ CO	CDCl ₃	CCl ₄	
$\alpha\text{Hiv } \text{C}_\gamma$	17.7	16.9	16.7	16.6	16.0
$\alpha\text{Hic } \text{C}_\gamma'$	17.9	17.3	17.0	17.0	16.0
l-Val C_γ	18.9	18.8	18.7	19.1	17.1
l-val C_γ'	19.3	18.8	18.7	19.3	17.1
d-val C_γ	19.5	19.0	19.2	19.5	17.1
d-val C_γ'	19.7	19.2	19.2	19.6	17.1
lac C_β	19.8	19.3	19.2	19.7	17.4
l-val C_β	31.0	29.6	28.4	27.7	27.3
d-val C_β	31.3	29.6	28.4	27.7	28.7
$\alpha\text{Hiv } \text{C}_\beta$	31.8	30.3	30.2	27.9	28.7
l-val C_α	59.8	58.5	58.8	59.0	66.4
d-val C_α	59.8	60.0	60.2	60.2	66.4
lac C_α	71.7	70.6	70.1	69.7	76.9
$\alpha\text{Hiv } \text{C}_\alpha$	79.7	78.8	78.4	77.9	85.8

Table 5 continued

resonance *	Solvent				calc.
	MeOH	Me ₂ CO	CDCl ₃	CCl ₄	
lac C ₀	171.4	170.0	169.8	170.2	
αHiv C ₀	172.4	170.4	170.4	169.5	
l-val C ₀	171.9	171.6	171.4	171.9	
d-val C ₀	173.0	171.6	172.1	171.0	

* distinction between d- and l-valine resonances is tentative.

two lower field resonances were tentatively assigned to the valine residues and the remaining two to the hydroxy acids; a similar argument was later used by Ohnishi and co-workers (1972) in their assignment of these carbonyl resonances. Assignment of the C_α , C_β and C_γ resonances was based on comparison with the predicted chemical shifts of these resonances in the various residues, obtained from calculations using the substituent parameters employed by Horsley and co-workers (1969, 1970) and by comparison with the ^1H resonance spectrum, bearing in mind the general correlation between the chemical shifts of ^{13}C nuclei and those of protons bonded to them (Emsley et al., 1965; Del Re et al., 1963; Horsley et al., 1970). In the cases of resonances of nuclei at corresponding positions in the d- and l-valine residues, and of chemically equivalent positions in the same residue, the lines fall closely together and a distinction was not readily made; tentatively, the higher-field resonances have been assigned to the l-valines in observance of the situation in the ^1H spectrum. These assignments are in agreement with those of Bystrov and co-workers (1972) and Ohnishi and co-workers (1972).

Interpretation:

The sensitivity of the molecular conformation to solvent changes, noted in the discussion of the ^1H spectrum (p. 15)

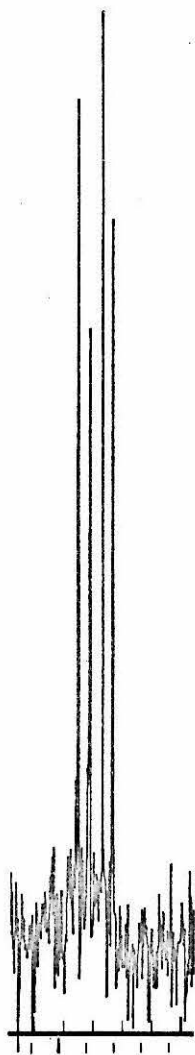
is manifested again in the spectrum of ^{13}C resonances. The range of methyl resonance positions, for example, is increased by nearly 50% as the dielectric constant of the solvent changes from 31.7 in MeOH to 2.2 in CCl_4 ; furthermore the observed range of these shifts (3.1 ppm) substantially exceeds both that calculated for the nuclei concerned (1.4 ppm) and that observed for sequence analogues of the valinomycin residues (1.8 ppm, Ohnishi *et al.*, 1972). Thus on incorporation into the tertiary structure of the molecule, the various residues experience effects which confer on each of them particular chemical shifts, and these must be related to the conformation of the species. Decrease of solvent polarity, therefore, leads to conformation changes in the molecule which increase the uniqueness of each methyl group's environment.

Alteration of solvent polarity also affects the positions of the C_α and C_β resonances, of which the former are the more informative by virtue of their reasonably facile assignment. The resonances of the C_α nuclei in the hydroxy acid residues are more profoundly shifted than those of the amino acid C_α 's; undergoing a continuous progression to higher field as the dielectric constant decreases, while their amino acid counterparts are largely unaffected, and in some cases shifted to lower field. These shift differences could be attributed to decreased polarization of the

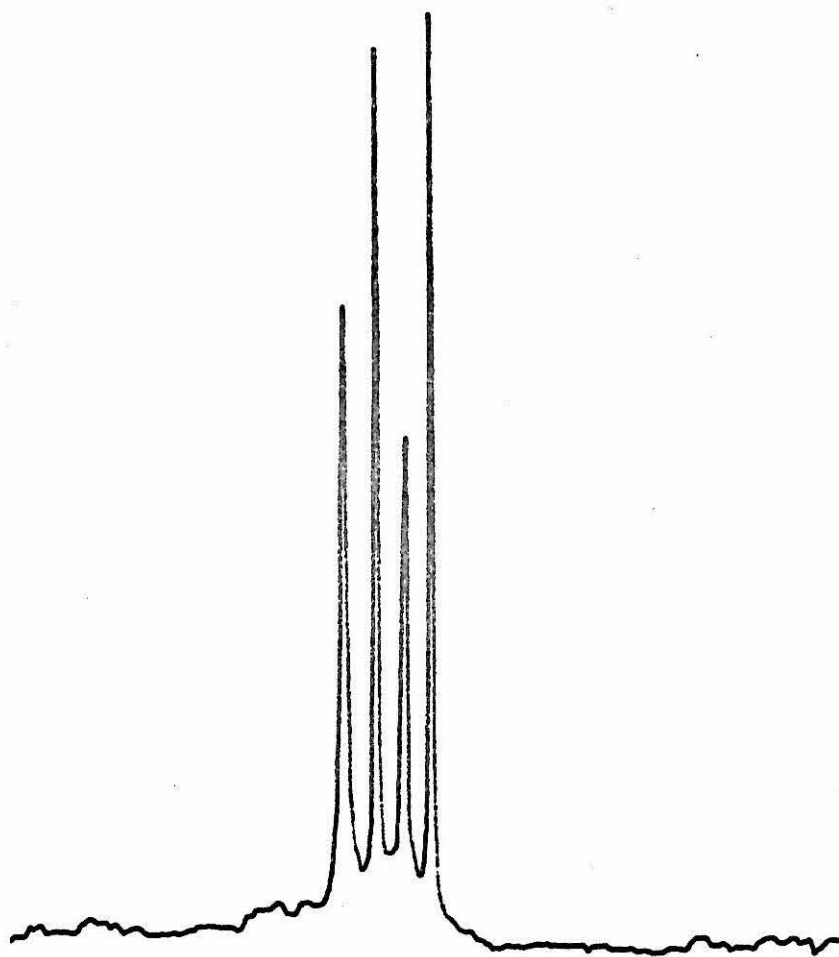
amide carbonyls resulting from loss of interaction with polar solvent molecules, an interpretation requiring a structure wherein the ester carbonyls are excluded from such an interaction with solvent. Direct solvent effects on methynyl ^{13}C resonances are expected to be negligible (Batchelor et al., 1972) and this, taken in conjunction with consideration of the carbonyl ^{13}C shifts, indicates that other effects, probably arising from conformation changes, are also important.

Applying the peak-height argument offered earlier (p.37) and by Ohnishi et al. (1972), for the identification of the ester and amide carbonyl resonances, it is apparent that at least in the two solvents CD_3OD and CCl_4 , the order of the resonances in the spectrum is substantially altered. Ohnishi et al. (1972) found that in CD_3OD the order was, from lowest field: ester, amide, ester, amide, and apparently this order is maintained in CDCl_3 (Fig. 8a); in CCl_4 , however, the order is clearly reversed (Fig. 8b). In considering the magnitudes of these chemical shift changes (Table 5), it was observed that the carbonyl resonances of the valines were shifted to lower field by at least two ppm on change of solvent from CCl_4 to MeOH , while the corresponding resonances of the hydroxy acids were shifted to lower field by only half that amount. In the case of the C_α resonances, the situation is the opposite: on replacement of CCl_4

Figure 8: Carbonyl ^{13}C resonances in (left) CDCl_3
and (right) CCl_4 .



a



b

by MeOH, one of the valine resonances was shifted less than one ppm to lower field and the other in fact moved 0.4 ppm to higher field, while the C_{α} resonances of the hydroxy acids were shifted to lower field by an average of 1.9 ppm each. Assuming the shifts of the carbonyl resonances to be governed largely by π -bond polarity (Stothers and Laubur, 1964; Maciel and Natterstad. 1965; Maciel, 1965), if the inductive effect of the carbonyl group on the C_{α} electron density were the chief factor in determining the C_{α} chemical shift, one would expect the C_{α} shifts to follow those of the carbonyl carbons of the same residue. Since they do not, it is probable that other effects are significant contributors to the observed chemical shifts, and these are likely to arise from conformational differences such as reorientation of the atom in question with respect to a nearby anisotropic group, for example another carbonyl as has been suggested in the 1H spectrum by Ohnishi and Urry (1969). Evidence for structural alteration on alteration of the medium, then, is found throughout the ^{13}C spectrum as well as the 1H spectrum.

The chemical shifts of the ^{13}C resonances in the $VM \cdot K^+$ complex are given for various solvents in Table 6, and were assigned by comparison to the spectrum of free valinomycin. The resonances of the side-chain carbons experience small shifts on complex formation, with almost all moving by less than

Table 6

Solvent Dependence of VM·K⁺ ¹³C Chemical Shifts (ppm from TMS)

resonance*	Solvent			
	MeOH	Me ₂ CO	CDCl ₃	CCl ₄
αHiv C _α	17.3	17.1	17.0	16.9
αHiv C _α '	17.9	17.8	17.6	17.4
l-val C _α	19.4	19.1	19.0	19.0
d-val C _α	19.4	19.3	19.3	19.3
d-val C _α '	20.6	20.4	20.3	20.5
l-val C _α	29.8	29.1	28.3	28.1
d-val C _β	29.8	29.1	28.4	28.2
αHiv C _β	31.9	29.1	30.3	30.0
l-val C _α	63.0	62.5	61.6	61.4
d-val C _α	63.2	62.7	61.8	61.6
lac C _α	72.6	72.3	71.1	70.9
αHiv C _α	80.9	80.5	78.0	79.1

Table 6 continued

resonance [*]	Solvent			
	MeOH	Me ₂ CO	CDCl ₃	CCl ₄
lac C ₀	172.7	172.0	170.6	171.4
αHiv C ₀	174.1	173.1	172.0	172.8
l-val C ₀	176.5	176.0	174.4	172.9
d-val C ₀	176.9	176.6	175.0	173.6

* distinction between d- and l-valine resonances is tentative.

one ppm to lower field, while the shifts of the backbone carbon resonances are more significant. The participation of the ester carbonyls in the interaction with the ion is clearly evidenced by their substantial shift to lower field on complexation, in accordance with increased polarization of those moieties by the ion's electric field. In addition, the ^{13}C resonances of the amide carbonyls also experience shifts to lower field, which have been attributed (Bystrov et al., 1972) to participation of those groups in an ion-dipole interaction with K^+ . However, inspection of models produced from spectroscopic (Ivanov et al., 1969; Macmurchie, unpublished) and X-ray (Pinkerton et al., 1969) studies shows that the amide carbonyls are to be found in an orientation perpendicular to the metal ion, one in which the ion might not be expected to exert a great polarizing influence (Millett and Raftery, 1972). Accordingly, it is reasonable to suggest that enhanced intramolecular hydrogen bonding (Ivanov et al., 1969; Ohnishi and Urry, 1969) may be important in producing these shifts. Such an interpretation would be consistent with the observation (Maciel and Natterstad, 1965) of the significance of H-bonding in determining ^{13}C chemical shifts in carbonyl groups.

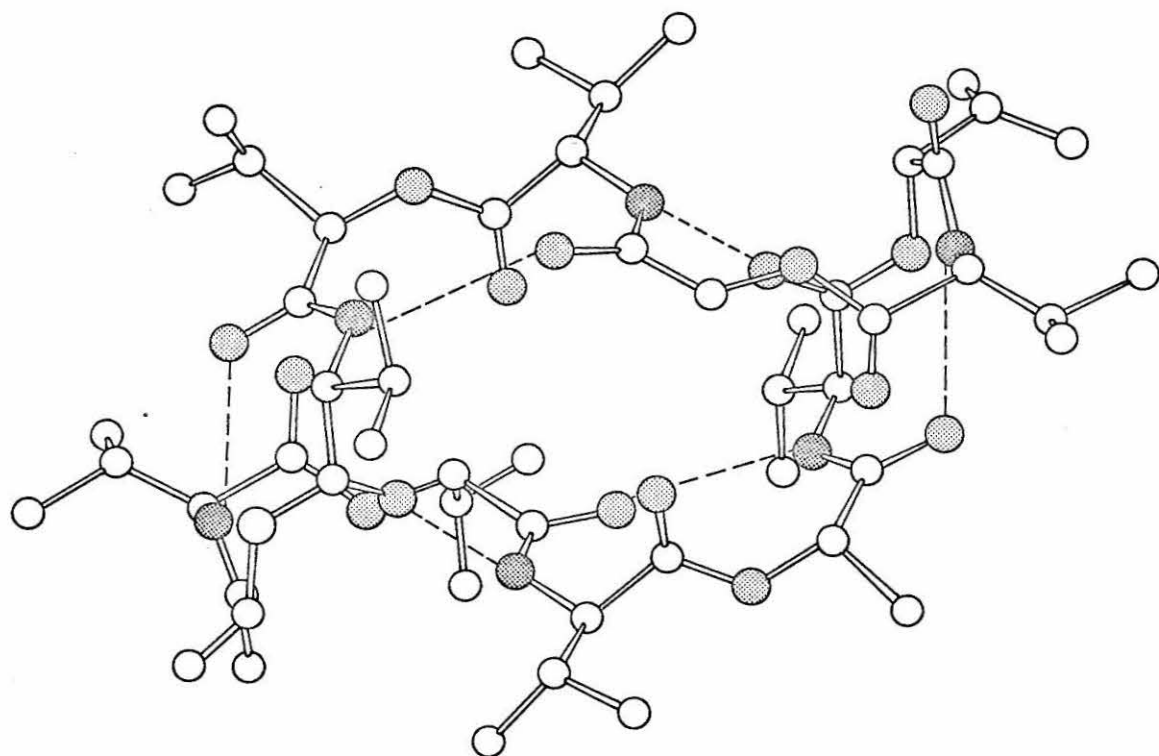
It is seen, therefore, that the ^{13}C nmr spectrum is informative in two important respects: direct identification

of the groups involved in complexation, the valine carbonyls, is obtained, and the greater range and simplicity of the ^{13}C spectrum allows the dependence of the molecular conformation on its medium to be more readily observed.

Conformational Processes in Valinomycin:

In the preceeding pages, it has been emphasized that conformation changes take place in valinomycin on alteration of its medium and on formation of a complex with K^+ ; in the case of the K^+ complex, X-ray analysis of the crystalline material (Pinkerton et al., 1969) has confirmed the structure first proposed (Ivanov et al., 1969) on the basis of spectroscopic studies, and has revealed the three-fold symmetry that seemed apparent from the sequence and the simplicity of the nmr spectra. The structure of the antibiotic when not complexing a cation, although obviously not identical to that of the complex, was generally assumed (Ivanov et al., 1969, Pinkerton et al., 1969) to have a similar configuration and H-bonding network. The recent publication (Duax et al., 1972) of an X-ray analysis of the crystal structure of the free species is therefore of great interest, particularly in that this structure (Fig. 9) is entirely asymmetric and involves a hydrogen-bonding arrangement not previously suspected for valinomycin. Inspection of the structure proposed by Duax and co-workers indicates that various chemically identical parts of the molecule are in situations which could lead to non-equivalence of their magnetic environments. Remembering the effect of H-bonding on the ^{13}C resonances of carbonyl groups (Maciel and Savitsky, 1964; Maciel and Natterstad, 1965), one might expect to distinguish on this basis among, for example, the resonances of the lactate carbonyls, of which two are hydrogen-bonded in the Duax structure and one is not. Similar distinctions can be made

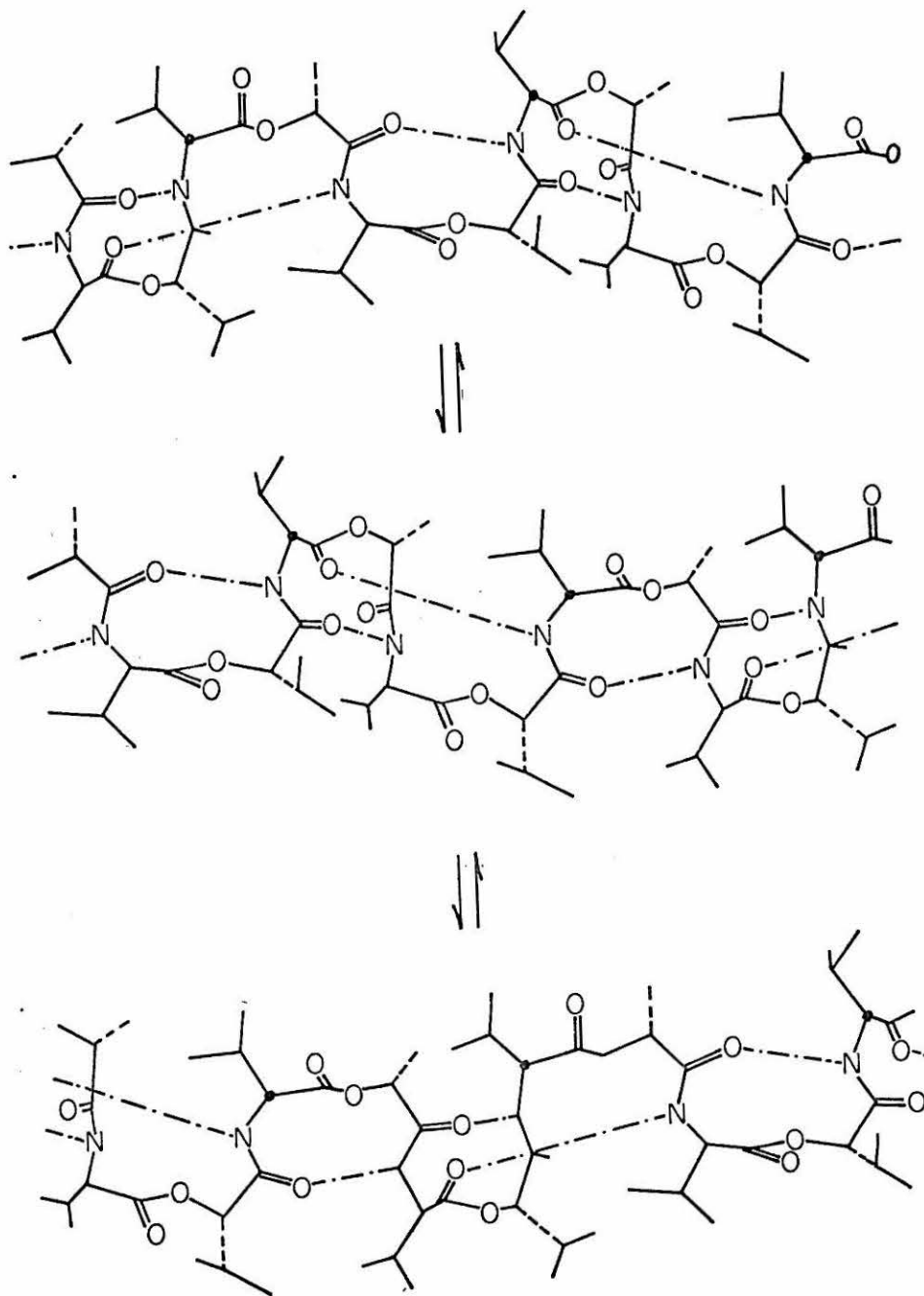
Figure 9: Crystal structure of valinomycin (Duax
et al., 1972); dark shading: nitrogen; light
shading: oxygen.



among the other chemically identical carbonyl groups, and in all one could predict the occurrence of eight resonances in the carbonyl region, in contrast to the four that were observed. Other chemically identical nuclei, such as the lactate C_{α} 's are also found in environments that have great potential for magnetic non-equivalence, by virtue of their differing orientation with respect to nearby anisotropic groups, specifically the carbonyls of the d-valines. Considering the proton spectrum, the efforts of Bystrov and co-workers (1969) have demonstrated the dependence of the coupling constant $^3J_{HNCH}$ on the conformational angle ϕ (Fig. 2, eqn. 2). Estimation of these angles in a molecular model of the Duax structure (Fig. 9) leads to the prediction of three doublets arising from the l-valine amides, with coupling constants of approximately 1 Hz, 7 Hz, and 10 Hz, and three from the d-valine amide protons, with couplings of 3 Hz, 7 Hz and 10 Hz. Similarly, using equation (1), the α Hiv α -protons would be expected to produce two doublets with large couplings, $^3J_{\alpha\beta} \simeq 9$ Hz, and one with $^3J_{\alpha\beta} \simeq 3$ Hz. Since, even at the highest fields employed, only one doublet is seen for each of these chemically identical atoms, there must be additional features to the solution conformation of free valinomycin that are not revealed in the crystal structure.

It is possible, of course, that the configuration of the molecule in solution is not at all related to that in the crystal form, but it seems more attractive to reconcile both sets of data in a model requiring the antibiotic to undergo a continual exchange of conformations, as illustrated in Fig. 10. Bearing in mind that the "ends" of the diagrams are to be thought of as joined, it is easily seen

Figure 10: Conformational equilibrium proposed for asymmetric structure in solution; d-valine residues identified by emphasized C_{α} .

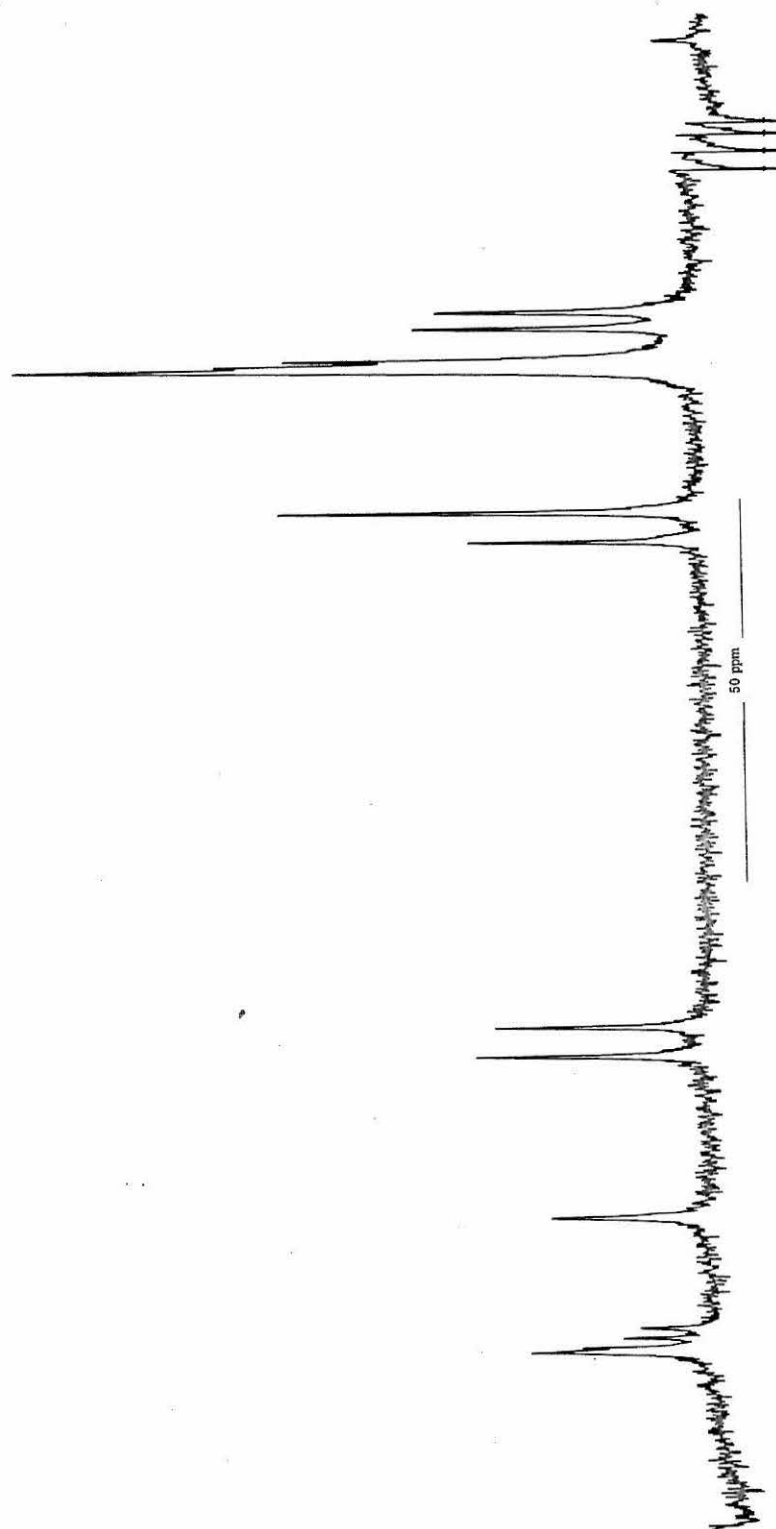


that they are identical in overall conformation, but that any individual atom, or group of atoms, finds itself in a different environment in each. If the molecule were to equilibrate rapidly among three structures such as these, an averaged nmr spectrum, similar to that observed, could result.

In order to determine kinetic parameters from nmr spectra, it is necessary to know the resonance frequency of a nucleus in each site in which it may be found, and this in turn requires obtaining a spectrum in the slow exchange limit, i.e., when $\tau \gg (2\pi \Delta\nu)^{-1}$ where τ is the time constant for exchange and $\Delta\nu$ is the difference in resonance positions, in Hz, of the sites involved. This limit would be most readily attained in the ^{13}C spectrum, considering its large range of chemical shifts, but that spectrum when recorded at the highest field and lowest temperature available (52 Kgauss and -50°C , respectively, Fig. 11) is not one characteristic of that type of exchange. Nevertheless, examination of the line widths in the ^1H spectrum indicates that an exchange process may indeed be taking place; particular attention is drawn to the doublets produced by the β -protons of the αHiv and lactate residues. From the spin-spin coupling constants of these resonances (2.8 Hz and 6.8 Hz, respectively) it is apparent that the αHiv has restricted rotation about the $\text{C}_\alpha\text{-C}_\beta$ bond ($\phi = +123^\circ$, Table 2) whereas the lactate methyl group is likely to be rotating freely. This being the case, one would expect the resonance of the lactate protons to be at least as sharp as that of the αHiv proton, whereas, in fact,

Figure 11: ^{13}C nmr spectrum of valinomycin in CDCl_3 at -50°C , 55 MHz. ^1H noise decoupled, spectrum width 5 KHz; inverted signals are images of carbonyl resonances at lower field.

Hz
↑
1



it is broader by more than 20% at ambient temperature and 100 MHz ($\Delta\nu = 1.72$ Hz and 1.41 Hz, respectively; Fig. 12); it is also observed that the width of the lactate methyl resonance is less when the complex with K^+ is formed than in the absence of the ion (Fig. 13). These results would be produced by a situation in which the molecule experiences rapid exchange among conformations having greater chemical shift differences for the lactate than the α Hiv residues, and in which the exchange is eliminated by formation of a symmetric complex structure. In addition the lactate methyl line-width exhibits a field and temperature dependence (Table 7) that is compatible with such a scheme.

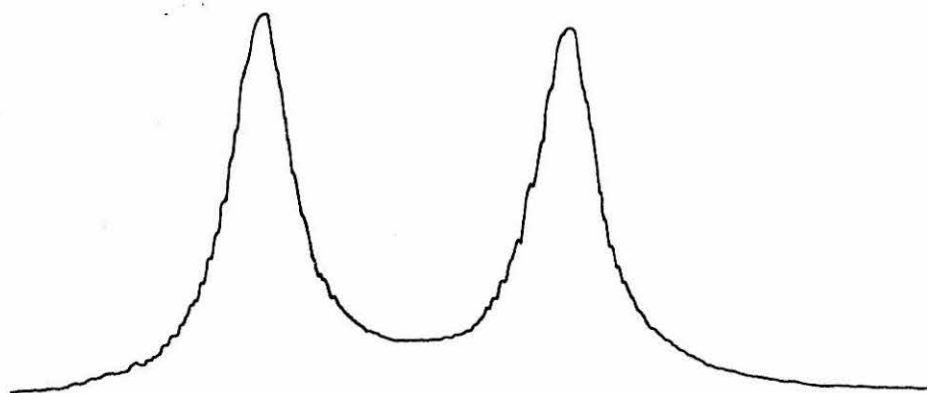
Conformation changes associated with complex formation also lead to exchange phenomena, as has been observed in the 1H spectrum by Haynes et al. (1969). In the ^{13}C spectrum, less-than-saturating amounts of K^+ produce different exchange conditions depending on the solvent employed; Fig. 14 compares these spectra in methanol, acetone and CCl_4 . Taking the line widths of the hydroxy acid C_α resonances and employing the relation for reasonably fast exchange (Pople et al., 1959)

$$\frac{1}{T_2} = \frac{p_A}{T_{2A}} + \frac{p_B}{T_{2B}} + p_A^2 p_B^2 (\omega_A - \omega_B)^2 (\tau_A + \tau_B) \quad 3$$

one may calculate the average turn-around time for the exchange process in methanol and acetone (Table 8); in CCl_4 these processes are too slow to be determined by this method. These rates follow

Figure 12: ^1H resonances in CDCl_3 of (top) lactate CH_3 and (bottom) α -hydroxy-iso-valerate C_αH .

-58-



10 Hz

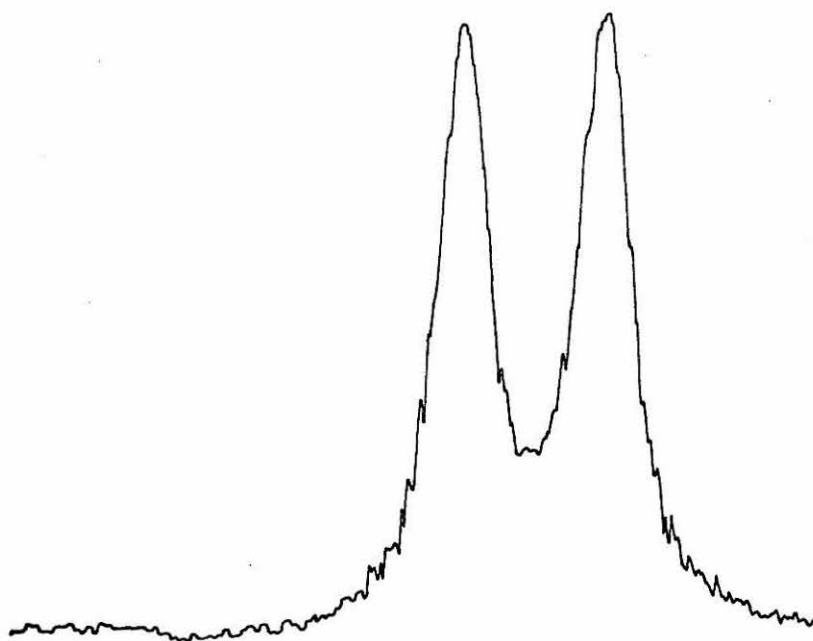


Figure 13: ^1H resonances of lactate CH_3 in CDCl_3 of (left) $\text{VM}\cdot\text{K}^+$ and (right) VM .

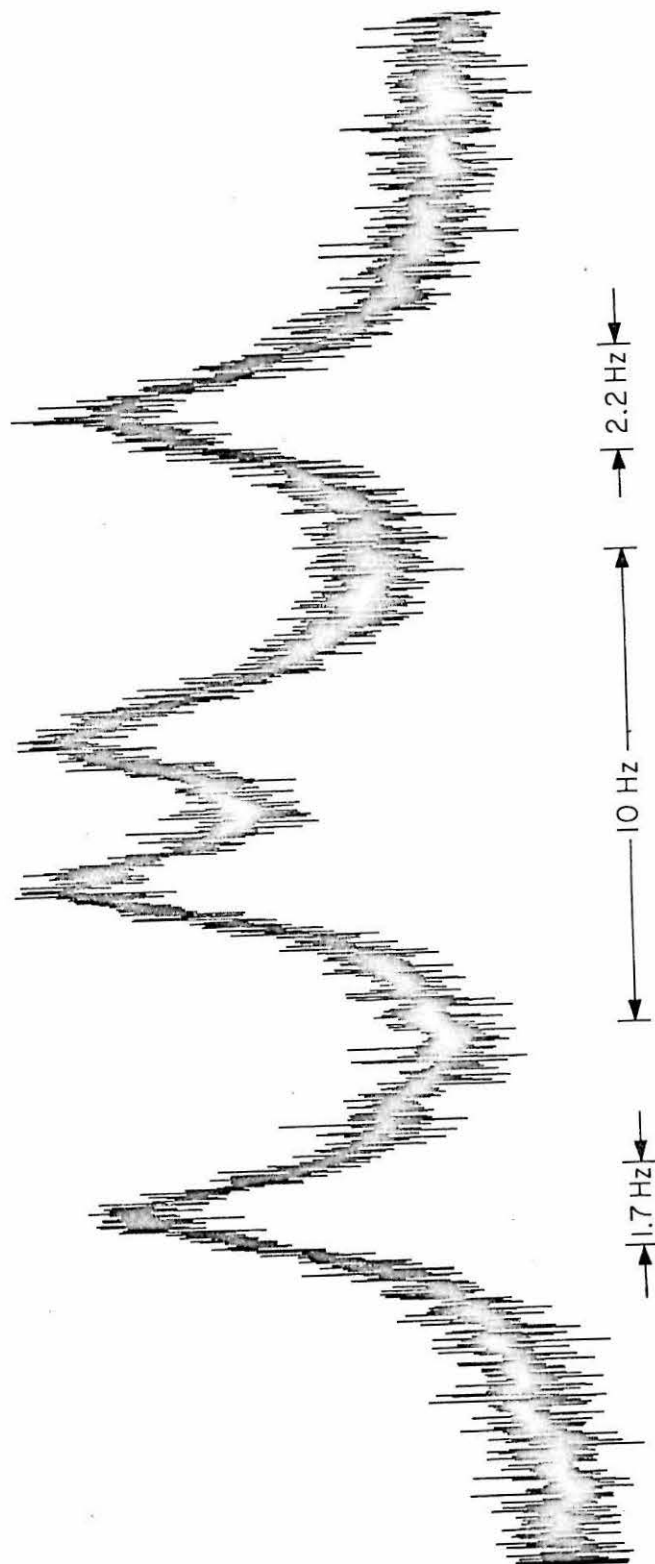


Table 7

Temperature and Field Dependence of Lactate C^1H_3 Line Width

Field (Kgauss)	$\Delta\nu(\text{lac})$	$\Delta\nu$ (TMS)	corrected $\Delta\nu$ (lac)
14.1	$1.58 \pm .06 \text{ Hz}$	$0.59 \pm .06 \text{ Hz}$	$0.99 \pm .13 \text{ Hz}$
23.5	$1.71 \pm .02$	$0.62 \pm .04$	$1.09 \pm .06$
51.7	$2.27 \pm .10$	$0.74 \pm .05$	$1.53 \pm .15$

Temperature

32° C	$1.71 \pm .02 \text{ Hz}$	$0.62 \pm .04$	$1.09 \pm .06 \text{ Hz}$
0°	$2.83 \pm .10$	$0.39 \pm .02$	$2.44 \pm .12$
-20°	$3.55 \pm .10$	$0.58 \pm .05$	$2.97 \pm .15$

Spectra were recorded with minimum filtering; data are averages of four measurements.

Figure 14: ^{13}C nmr spectra of $\text{VM}:\text{K}^+ = 10:6$ in
a) MeOH , b) Me_2CO , c) CCl_4 .

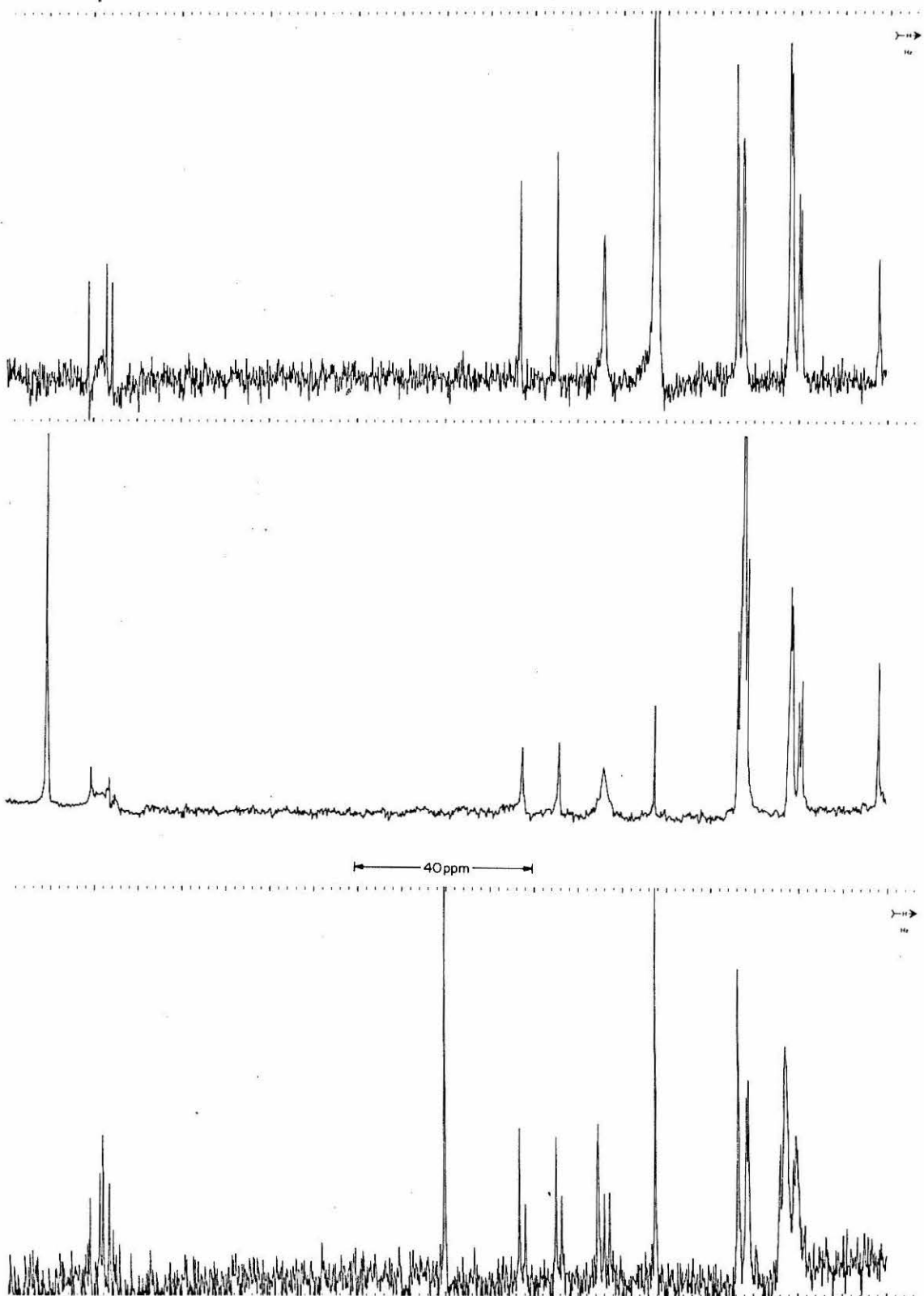


Table 8

VM·K⁺ VM + K⁺ Exchange Rates:

C_α Resonances

$$\frac{1}{T_2'} = \frac{p_A}{T_{2A}} + \frac{p_B}{T_{2B}} + p_A^2 p_B^2 (\omega_A - \omega_B)^2 (\tau_A + \tau_B)$$

	K ⁺ :VM	$\Delta\tau$ (Hz)		$\Delta\omega/2\pi$ (Hz)	
		lactate	α Hiv	lactate	α Hiv
MeOH	0:1	3.5 ± 0.5	3.0 ± 0.5		
	2:5	8.0	8.0	26 ± 1	28 ± 1
	1:1	4.0	4.0		

$$\tau_A + \tau_B = (2.7 \pm 0.3) \times 10^{-3} \text{ sec}$$

Me₂CO

	0:1	6.0 ± 0.5	7.0 ± 0.5		
	2:5	10.0	12.0	22 ± 1	32 ± 1
	1:1	5.0	4.0		

$$\tau_A + \tau_B = (3.9 \pm .05) \times 10^{-3} \text{ sec}$$

the solubilities of KSCN in those solvents and, as the rate of ion uptake is likely to be extremely rapid (cf: $k_{ON} = 3 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$ for monactin- Na^+ complex, Eigen and Winkler, 1971), it is probable that the exchange rates are determined by the propensity of the ion to leave the antibiotic in the various solvents.

Discussion

The exact means whereby valinomycin, and other ionophores, mediates the passage of an ion through a non-polar, lipid barrier has been the subject of some discussion: some authors (Ohnishi and Urry, 1969) have suggested a "pore" mechanism, in which the molecules stack on top of one another so as to present a hydrophilic channel through a membrane, while others (Haynes et al., 1969; Pinkerton et al., 1969; Tosteson, 1971) have argued for an individual carrier, shuttling back and forth, picking an ion up at one side and releasing it at the other. It has also been suggested (Haynes et al., 1969; Duax et al., 1972) that aspects of both processes are involved, and that while acting independently of other ionophores, a single one may remain relatively fixed in the membrane and allow ions to pass through it to the opposite side. It is quite possible, of course, that different ionophores act in different ways, and since none of these compounds have been detected in nervous tissue (indeed, the "crown ethers" (Pederson, 1967) are not natural products at all) that the relationship that they bear to the mechanisms of nervous conduction may seem obscure. Nevertheless, they serve as useful models and it is to be hoped that their study may lead to at least some understanding of more complex systems.

In the case of valinomycin, the structure proposed here, later modified to concur with the observations of Ivanov et al.

(1969) has characteristics appropriate to a single-carrier species. The iso-propyl and methyl side chains effectively envelop the ion and its surrounding carbonyls, providing the complex with an exterior that is compatible with the hydrophobic environment through which the ion must pass. The six ester carbonyls, octahedrally arranged at a distance of about 3 Å, provide the K^+ ion with a local environment similar to that which it experiences in aqueous solution, in which the hydration number is four or five (Bell, 1958); thus the complex is a "water-drop" in non-aqueous media, analogous to the "oil-drop" model in which many proteins have been cast.

The probability of the molecules acting independently of one another is heightened by the facts that the complex structure does not allow intermolecular H-bonding in the axial direction, that the molecule has no ionizable groups, and that hydrophobic interactions are not likely to be important in the lipid environment. These observations are strengthened by the lack of concentration effects in the nmr spectra reported by Haynes and co-workers (1969) as well as in this work (p.) and also by the observation (Pinkerton et al., 1969) that the rate of valinomycin-mediated equilibration of ion concentrations in aqueous solutions separated by chloroform was highest at less than saturating ion concentrations. This latter observation was interpreted as meaning that the return of an empty valinomycin shuttle to the high concentration

side was necessary, and was prevented by high K^+ concentrations on the receiving side; thus a mechanism of independent carrier molecules was implicated.

The outstanding difference between the $VM \cdot K^+$ structure proposed on the basis of early work in this thesis (Fig. 5) and that proposed by Ivanov and co-workers (1969, Fig. 6) lies in the disposition of the amide $>C=O$ and $>N-H$ moieties. In Figure 5, these are seen to be of two distinct types, the lactyl amides having their carbonyl groups relatively exposed and their amide protons more or less buried, while the iso-valeryl amides are to be found with their carbonyls secluded and their amide protons more exposed to solvent. This arrangement is in accordance with the observation of different solvation properties for the two types of amide protons (p. 18) and observes the convention of amide group planarity. Completion of the intramolecular H-bonding network suggested by Ivanov and co-workers (1969) slightly compacts the $VM \cdot K^+$ structure, makes the distinction between the amide groups more subtle, and judging from carefully constructed space-filling models, causes the amides to be substantially deformed from a planar configuration. This possibility has not been mentioned by Ivanov (1969) or Pinkerton (1969), but has been taken as a necessity in conformational energy calculations (Mayers and Urry, 1972). Prior to publication of the free valinomycin structure reported by Duax and

co-workers (1972) most others had assumed the free species to resemble closely the complex structure (cf. Fig. 6a and b). In this arrangement, the ester carbonyl oxygens would be free to withdraw slightly from the center of the cavity in the absence of K^+ and it appears possible for the amide groups to enjoy a planar configuration under those circumstances. A situation of this type could provide a mechanism for maintaining the carrier in a more open, or receptive, conformation before the arrival of the ion and allowing it to close about the ion as it transports it through the hydrophobic region.

While not denying the possibility of a carrier-diffusion mechanism for valinomycin action, Duax and co-workers (1972) have suggested that the asymmetric crystal structure confers upon valinomycin properties such that it "could be lodged in a membrane pore and act as a pump". In this context it is of particular importance to consider the possibility of different structures in solution and in the crystal state, or ways in which a single structure could give rise to these disparate observations. In interpreting their ORD data, Ivanov and co-workers (1969) favored a situation in which equilibrium existed between two conformers of greater and lesser symmetry, with the equilibrium constant changing according to solvent polarity; it is possible that the same results could arise from a single conformation, which itself altered with the polarity of the medium. Such a single conformation could still undergo periodic internal reorganiza-

tion of the type previously hypothesized (p. 55) and produce an averaged nmr spectrum of the type observed, but which varied with solvent polarity (pp. 15, 38).

As has already been mentioned, one cannot determine exchange rates from nmr spectra without knowledge of resonance positions in each state; however, an approximation may be useful. Variations in H-bonding for chemically identical moieties in the asymmetric structure have been pointed out (p. 50); for carbonyl groups, Maciel and Savitsky (1964) have found that H-bond formation results in shifts of the ^{13}C resonances to lower field by 3-7 ppm. At 52 Kgauss, 3 ppm = 166 Hz for ^{13}C , so taking a typical line width of $(2\pi T_2')^{-1} = 10$ Hz and assuming a line-width in the absence of exchange of $(2\pi T_2)^{-1} = 1$ Hz, one may calculate an exchange rate for the simplified case of two sites from Eqn. 3: this turns out to be $1.5 \times 10^4 \text{ sec}^{-1}$, and line broadening contributions from other sources would increase this figure. It is unlikely that treatment of an exchange as among three sites would lead to a significantly different exchange rate (K.L. Reed, personal communication). A rate of this magnitude seems prohibitively great, especially in comparison to the cyclohexane chair-chair interconversion process which has been measured, at -66.5°C , to be $1.2 \times 10^2 \text{ sec}^{-1}$ (Jensen et al., 1960). However, in the case of antamanide, a cyclic decapeptide ionophore, it has been reported (Faulstich et al., 1972) that the

molecule undergoes a conformational equilibrium with a time constant in the microsecond range, although the nature of the conformation change was unspecified; in view of this, an extremely rapid equilibration of asymmetric structures for valinomycin may not be totally unthinkable. Failing such a condition, one might suggest that in the crystal state packing forces may produce a slightly abnormal configuration, a situation that has been found in the crystal structures of smaller molecules (Benedetti et al., 1968; Ganis et al., 1970; Pedone et al., 1970).

However the apparent dilemma presented by the crystallographic and spectroscopic data is resolved, one must conclude that the molecule exhibits remarkable flexibility and this is almost certainly a significant factor in its mechanism of action. The data presented and reviewed here tend to favor a single carrier in a lipid membrane; indeed, if as rapid a conformational equilibrium as has been suggested is taking place, it is difficult to imagine a number of contiguous molecules acting so feverishly in concert. It seems likely that two separate sorts of process may be occurring at once as the carrier continuously traverses a membrane: first, an extremely rapid structural averaging, and second, an alteration in average structure as a function of medium polarity. This could result in a situation wherein valinomycin, approaching the more polar membrane boundary from the interior, adopts an average conformation

having its hydrophilic pocket more exposed while at the same time rapidly presenting various of its polar groups as initial attachment sites for an incoming ion. Such a scheme correlates well with a rapid sequential chelation mechanism (Eigen and Winkler, 1971). Having enclosed the K^+ ion, the carrier may then complete its task by diffusing across the membrane and releasing the ion on the other side.

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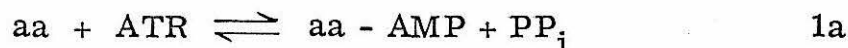
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PROPOSITION I

It is proposed to investigate the specificity of Ile-tRNA^{Ile} synthetase in its acylation of tRNA's and its deacylation of mischarged aminoacyl-tRNA's in methanol-water mixtures.

The precision of translation of the genetic message during the process of protein synthesis is widely remarked upon, and the efficiency with which aminoacyl-transfer ribonucleic acid (aa + tRNA) synthetases recognize their substrates in the catalysis of reactions 1a and 1b is very great. Despite the interest



in them, aa-tRNA synthetases remain poorly understood enzymes: only one has been crystallized (Chirikjian et al., 1972) and the molecular structure is not yet known. Some of the enzymes appear to be complexes of either identical (Chirikjian et al., 1972) or non-identical (Lapointe and Söll, 1972) subunits while others are single chains (Baldwin and Berg, 1956). Among the best known of these enzymes is E. coli Ile-tRNA^{Ile} synthetase (IRS, Baldwin and Berg, 1956) which has the interesting property, perhaps shared by other aa-tRNA synthetases, of possessing a

deacylation as well as an aminoacylation site (Eldred and Schimmel, 1972; Yarus, 1972a); it has been shown that this deacylation, reaction 2, which does not depend on inorganic phosphate (PP_i) or adenylic acid (AMP), is not the reverse process of aminoacylation, and it has been suggested as a mechanism for correction of mischarging errors (Schreier and Schimmel, 1972) which, in spite of the enzyme's renowned specificity, do occur. In addition to deacylating mischarged Val-tRNA^{Ile}, IRS will, at a slower rate, deacylate Ile-tRNA^{Ile} (Schreier and Schimmel, 1972). Mischarging apparently occurs by the binding of incorrect tRNA's to the aa-tRNA synthetase, rather than by transfer of an incorrect amino acid to the appropriate tRNA (Roe and Dudock, 1972; Yarus, 1972 a,b) and the exact means whereby the synthetase recognizes the appropriate tRNA is a subject of considerable interest. If the deacylation site has indeed evolved as an error-correcting mechanism, the deacylation of correctly charged Ile-tRNA^{Ile} could be regarded as being itself a mistake in eliminating a desired product; in that respect it would seem that the major specificities of the enzyme are complementary: the aminoacylation site does not transfer other than, in this case, isoleucine, and the deacylation site does not remove amino acids from other than tRNA^{Ile}.

It has been observed (Yarus, 1972b) that incubation of E. coli IRS with substrates in a medium containing 20% MeOH leads to

a vastly increased rate of mischarging of tRNA^{Phe}; this phenomenon has been attributed to loosening of the tRNA^{Phe} structure so that it resembles, at crucial points, the structure of tRNA^{Ile} and is recognized by the IRS acylation site. It is proposed to investigate the deacylation activity of the enzyme toward Val-tRNA^{Phe} under those conditions and compare it to the deacylation of Val-tRNA^{Ile}. If the tRNA^{Phe} structure in 20% MeOH does resemble that of tRNA^{Ile}, increased deacylation should result; conversely, if the addition of methanol so alters the tRNA^{Phe} structure, it might well cause the tRNA^{Ile} structure to be unrecognizable and so allow mischarged Val-tRNA^{Ile} to escape.

If these effects were observed, one would be encouraged to undertake a survey of aa-tRNA synthetases and their substrates, attempting to ascertain which could be made to resemble which others and from this to learn about the recognition process of the synthetase; if they were not observed one might seek alternative interpretations for the behavior of the enzyme in alcoholic solution.

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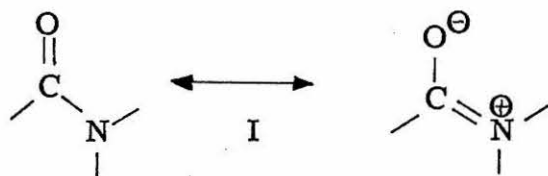
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PROPOSITION II

It is proposed to prepare and study the properties of cis- and trans-2-aminomethylcyclohexaneacetic acid lactams.

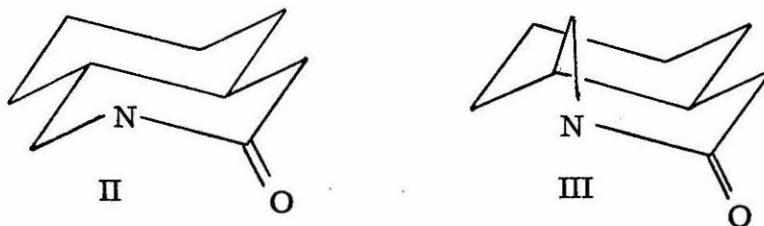
It appears likely that a number of interesting peptide compounds have at least some of their amide groups in conformations other than the planar arrangement that has been assumed for them for many years (Winkler and Dunitz, 1971). Among these are valinomycin (Mayers and Urry, 1972), enniatin B (Shemyakin et al., 1969) and antamanide (Faulstich et al., 1972); these observations have been made on the basis of model building and theoretical bases, in the first case, and from optical rotatory dispersion measurements in the latter two. It would be desirable to have other means of observing such situations, especially in solution, since the presence of deformed amide groups in several crystal structures has been attributed to intermolecular interactions in that state (Benedetti et al., 1968; Ganis et al., 1970; Pedone et al., 1970).

Conjugation of the type shown (I) is responsible for the



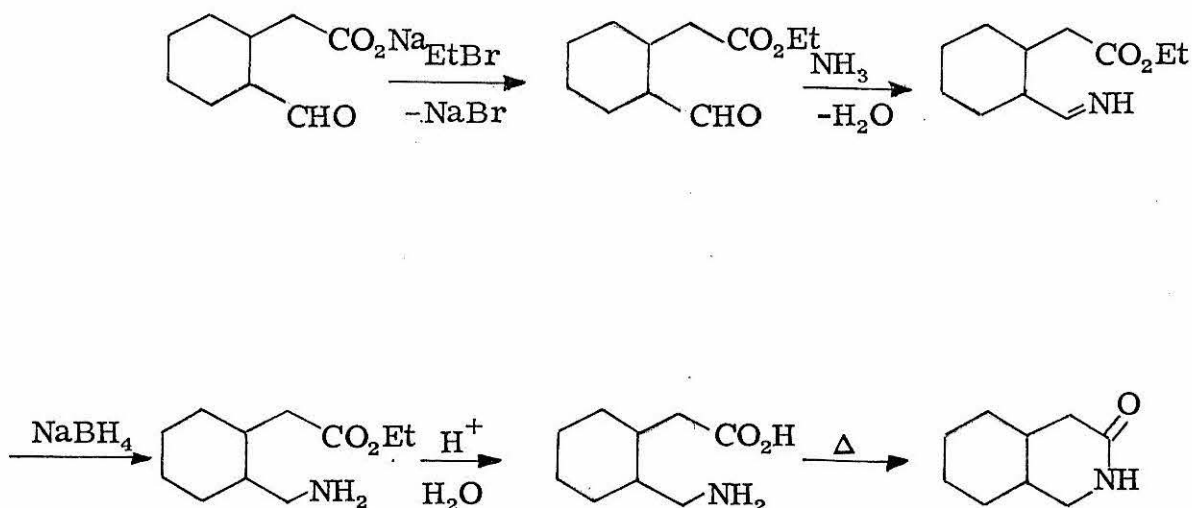
preference of amides for a planar configuration (Corey and Pauling, 1953); disruption of that resonance must accompany deformations from planarity. In the cases of conjugated ketones, it has been observed (Dhami and Stothers, 1964) that sterically induced deformations from planarity result in characteristic de-shielding of the carbonyl ^{13}C nmr signal, and that a good correlation may be made between chemical shift and deformation angle. Earlier, Lantebur (1962 a,b) had observed similar effects in the spectra of analines and nitrobenzenes. It would be reasonable to expect the $^{13}\text{C}=\text{O}$ resonances of amides to be effected in a like manner if those entities were deformed, and this could provide a valuable tool in the conformational analysis of small peptides.

In ascertaining whether such a deshielding (or, for that matter, any other) effect could be observed, one would wish to compare the spectral properties of simple compounds that differ as little as possible, save for their amide configuration and whose spectra are not complicated by effects such as chemical exchange. The pair of isomers II and III appear to be suitable: the amide group



of the trans isomer (II) would be substantially deformed, while that of the cis isomer (III) should enjoy planarity, and the molecules should remain quite rigid. Unfortunately, neither they nor their amino acid counterparts are available commercially, nor is there any mention of them in the reference literature (Chem. Abstracts).

Preparation of the desired compounds, however, should be readily accomplished from 2-formylcyclohexanecarboxylic acid:



In addition to the useful nmr data that should be available from these compounds, it is possible that stereochemical effects on the course of the reaction could be assayed by comparison of rates of formation and hydrolysis of the cis and trans compounds.

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PROPOSITION III

It is proposed to investigate the possibility of interspecies hybridization of ribosomal proteins and its use as a probe for ribosome function.

The study of enzyme-catalysed processes has developed from the contemplation of relatively simple and now well-understood proteins, e.g., lysozyme, through polymeric enzyme complexes, e.g., aspartate trans-carbamylase, and polyfunctional systems, e.g., the pyruvate dehydrogenase system, to ribosomes, which have been called (Kurland, 1972) the most complex objects yet to be investigated by biochemists. Consisting of two major subunits, which sediment at 30S and 50S in the case of bacterial matter, and containing upwards of 40 separate peptide chains averaging approximately 2×10^4 daltons each, intact ribosomes perform a series of catalytic and mechanical functions. Current theory (Lucas-Lenard and Lipmann, 1971) holds that these consist of binding an appropriate aminoacyl-transferRNA (aa-tRNA) according to instructions encoded on a messenger RNA (mRNA), catalysing the acylation of the aa-tRNA terminal amino group by a nascent peptide chain, translocating the now peptidyl-tRNA to a position from which it may donate the elongated peptide to a new aa-tRNA, and shifting one code group of three bases along the

mRNA. Energy for these processes is provided by GTP, for which a hydrolase is present.

The nature of the interactions among the ribosomal proteins is extremely complex: immunochemical studies (Stöffler et al., cited by Kurland, 1972a) indicate that none of them are completely buried in the ribosome, which is nevertheless relatively highly condensed; furthermore, the various proteins are assembled into the ribosomal structure in a methodical order (Pichon et al., 1972). The extent of interactions among the proteins is further indicated by the importance of various soluble "factors" that must be present in order for initiation, continuation and cessation of protein synthesis to take place. In bacterial ribosomes, investigations into protein interactions have dealt generally with the Escherichia coli 30S subunit, by reason of its ready availability and relative ease of reassembly (Taub et al., 1967; Nomura et al., 1969), frequently by omitting various proteins and studying the impaired function of the ribosome; this approach has the disadvantage that omission of a protein entirely causes too great an alteration in the structure, and all functions tend to be generally depressed (Kurland, 1972). Conversely, mutations in ribosomal genes have not produced viable bacteria (Flaks et al., 1966).

It is proposed to examine the species specificity of ribosomal protein interactions by growing E. coli in a radioactive medium, isolating and fractionating the chromosomal proteins and

adding them individually to mixtures of Bacillus stearothermophilus ribosomal proteins, from which intact ribosomes may be regenerated (Nomura and Erdmann, 1971; Erdmann et al., 1972); incorporation of radioactivity into the ribosomes should be readily detected. Assuming incorporation to take place, the hybrid ribosomes could be tested for activity in protein synthesis and could be expected to yield information bearing on the particular activities of the various proteins. If incorporation did not take place, it should be possible to gain some knowledge of the rejection process by adding proteins to the growing ribosomes in the assembly order suggested by Pichon and co-workers (1972) and observing the point at which reconstitution fails.

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PROPOSITION IV

It is proposed to investigate, by ^1H , ^{19}F and ^{31}P nmr the association of substrates with the galactosyl transferase (EC 2.4.1.22) in the presence and absence of the modifier protein, α -lactalbumin.

The galactosyl transferase EC 2.4.1.22 is a ubiquitous protein in mammalian systems, catalysing the transfer of galactose (gal) from UDP-gal to a number of different acceptors (Klee and Klee, 1972) including some glyco-proteins (McGuire et al., 1965). It has the interesting property of having its acceptor specificity altered from N-acetylglucosamine (NAG) to glucose (glc) in the presence of α -lactalbumin (α -LA), a common milk-whey protein whose function had long remained mysterious (Brew et al., 1967); this alteration appears to be accomplished by a mechanism which results in reduction both of K_M for each substrate and of K_i for substrate inhibition of the reactions. Thus at physiological conditions, α -LA lowers K_M for glucose to such an extent that it may be utilized effectively, and while also lowering K_M for NAG, simultaneously lowers K_i for NAG so that N-acetyllactosamine (NAL) synthesis becomes effectively inhibited (Morrison and Ebner, 1971c). The exact mechanism of the specificity alteration, however, remains unknown not least because the association of the

transferase and α -LA (lactase synthetase A and B proteins, respectively) is very weak, takes place only in the presence of glucose and produces no noticeable conformation changes (Klee and Klee, 1972). It has been shown that the binding of substrates, as well, perhaps, as the release of products, proceeds by an ordered mechanism, both in the case of lactose and NAL synthesis (Morrison and Ebner, 1971 a, b); the order of substrate and prosthetic group assembly on the enzyme is: 1) Mn^{+2} , 2) UDP-gal, 3) glc or NAG, [4) α -LA, if present]. It is conjectured that the disaccharide product departs before UDP (Morrison and Ebner, 1971b).

Knowledge of the spacial relationships among these components of the active complex would certainly prove useful in understanding the mechanism of both the catalytic and regulatory aspects of this enzyme system. It is proposed to take advantage of the requirement of the enzyme for the paramagnetic ion Mn^{+2} in conjunction with techniques developed by Cohn and Mildvan (Cohn and Leigh, 1962; Mildvan and Cohn, 1963, 1970) in attempts to ascertain: a) whether the binding of each substrate is equally effective in denying solvent H_2O access to the paramagnetic centre and b) the arrangement of the substrates around the Mn^{+2} ion.

The association of a paramagnetic ion with a macromolecule in solution produces a substantial increase in the rotational

correlation time of the ion, allowing it to affect more strongly the nuclear relaxation of associated H₂O molecules (Cohn and Reuben, 1970). The observed relaxation enhancement depends upon the number of solvent molecules associated with the paramagnetic centre as well as upon the average residence time in association according to the relation (Luz and Meiboom, 1964)

$$1/T_1 - 1/T_1^0 \equiv 1/T_{1p} = n \frac{[M]}{[L]} \frac{1}{(T_{1M} + \tau_M)}$$

where $n \equiv$ number of ligands in the complex

$[M]/[L] \equiv$ ratio of ion to ligand concentrations

$T_{1M} \equiv$ longitudinal relaxation time in the complex

$\tau_M \equiv$ residence time in the complex.

Since Mn^{+2} is necessary for the galactosyl transferase activity, it is likely that the substrates bind to the enzyme in such a way that solvent accessibility to the ion is altered in their presence, either by displacing H₂O from the hydration sphere of the ion or by preventing ready diffusion to and from it; further, depending upon the arrangement of the substrates, they may not be equally effective in this respect. Comparison of ¹H₂O longitudinal relaxation rates could be expected to produce useful information in this area. N-acetylglucosamine being a substrate for the reaction, it is highly likely that N-trifluoroacetylglucosamine (TFAG) also be accepted by the enzyme, and it would be of interest to

ascertain the effect of the Mn^{+2} on relaxation of the ^{19}F nuclei, as well as on the ^{31}P nuclei of the UDP-gal moiety; it should then be possible to relate the efficiency of relaxation to the distance of the nucleus in question from the paramagnetic species (Mildvan et al., 1971).

The glucose analogue, α -methyl-D-glucoside does not act as an acceptor in the lactose synthetase reaction (Babad and Hassid, 1966) and it is reasonable to suppose that it is bound to the enzyme but does not react; this could be easily ascertained. Assuming such a condition, it would be desirable to employ it and α -methyl-NAG or α -methyl-TFAG as inhibitors in the proposed research, in order to produce a stable complex for nmr study.

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PROPOSITION V

It is proposed to rig a sailing boat with lateral mast angle adjustment and to ascertain, by trials against a similar hull with fixed rigging, whether an improvement in performance can be realized.

The forces produced by the wind on a sailing boat may be considered in terms of a force per unit sail area (F_0) producing components in the foreward, lateral and vertical directions (Fig. 1), characterized by angles θ (to the vertical) and ϕ (to the wind direction); ϕ is not constant, but related to θ and the angle of the boom with respect to the boat, ϕ' :

$$\sin \phi = \sin \phi' \cos \theta$$

The total force on the sail is given by

$$F = F_0 A$$

where A is the projected sail area:

$$A = A' \cos \theta$$

$$A' = \text{actual sail area.}$$

The force driving the boat, F_D , is seen to be

$$F_D = F \sin \phi \cos \theta = F_0 A' \sin \phi' \cos^3 \theta.$$

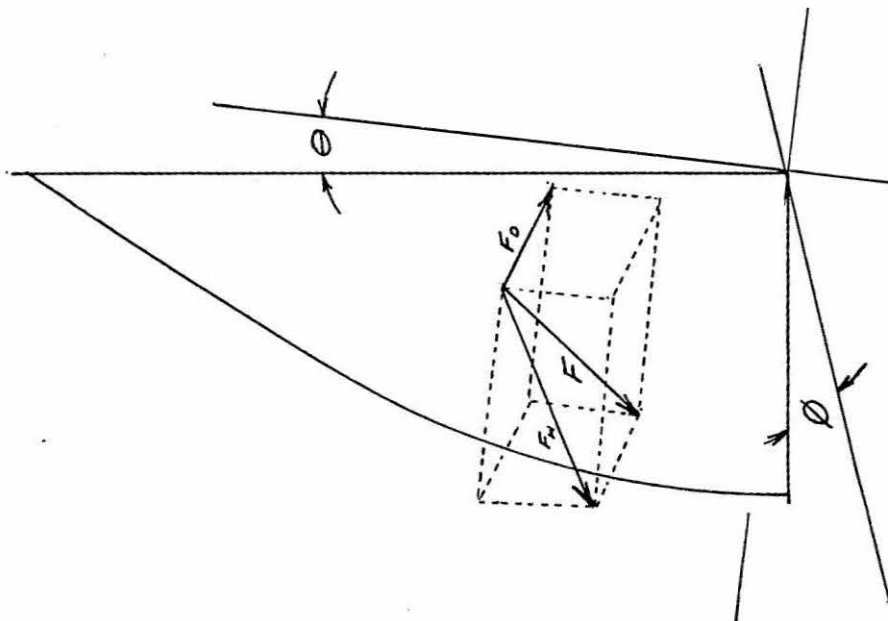


Fig. 1

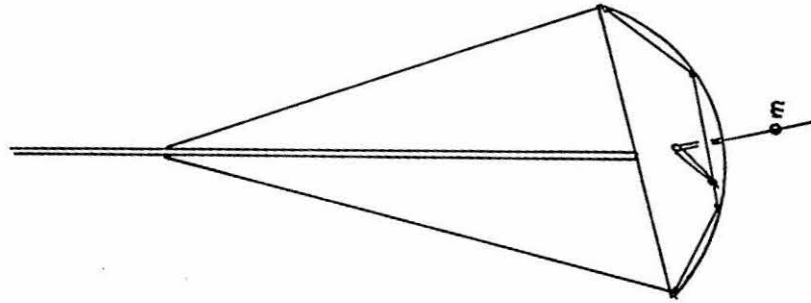


Fig. 2

Heeling (increase in θ) is one of the major causes of drag in sailing craft (Marchaj, 1964) and is produced by the heeling force F_H :

$$F_H = F \sin \phi \sin \theta = F_0 A' \cos \theta (1 - \sin^2 \phi \cos^2 \phi)^{1/2}$$

which acts through a moment arm L_H to the dynamic centre of the boat; resistance to heel is provided by displacement of the centre of mass from beneath the dynamic center, acting through its own moment arm, L_R . The righting moment at equilibrium

$$\Gamma_R = mL_R \sin \theta' = F_H L_H = \Gamma_H .$$

Obviously there is no righting moment at $\theta' = 0$; nevertheless, it is desirable to minimize θ for maximum driving force.

It is proposed to rig the port and starboard shrouds of a small boat to a common eccentric lever (Fig. 2) instead of to chainplates, and to take up the weather shrouds (simultaneously releasing the lee shrouds) on coming onto a new tack. Consider an alteration in mast angle from $\theta = 20^\circ$ to $\theta = 10^\circ$, for which an adjustment of a few inches would suffice. The change in driving force, for $\phi = 30^\circ$ (a typical angle) is readily calculated to be an increase of 9.6%; the change in heeling force on the other hand, increases by only 0.7%, i.e., friction losses to increased heel

are very small. Assuming friction losses to increase as the square of the speed, one expects on this basis a speed increase of over 4%, which should easily be observable.

Reference

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